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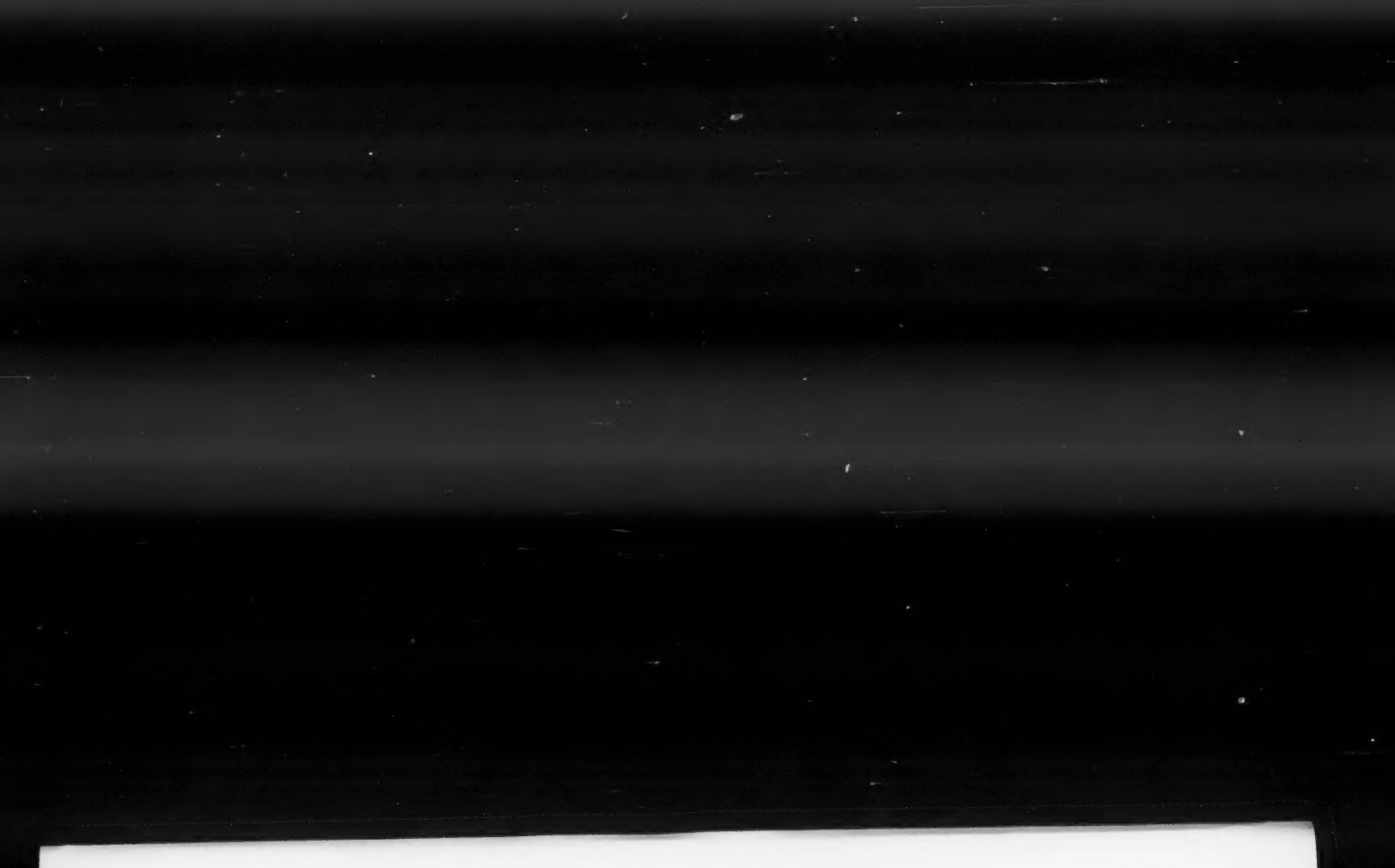

From the Physiological Department, Faculty of Medicine (Karolinska Institutet) and the Laboratory of Clinical Chemistry, Karolinska Sjukhuset, Stockholm.

Inactivation of Substance P by Proteolytic Enzymes.

By

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Received 7 March 1955.

Substance P, being a biologically active polypeptide, is inactivated by proteolytic enzymes. EULER (1936) showed, that the substance P activity is rapidly destroyed after incubation with glycerin extracts of pancreas, where trypsin was supposed to be the active principle; this has been confirmed by FISCHER and VOGT (1950). Substance P is also inactivated by extracts of intestinal muscle and brain tissue (GULLBERING 1943). Precipitation of intestinal muscle with acetone inactivated a solution of substance P by 50 per cent in 20 min. at pH 6.8 and 38° C. Extracts of brain tissue were even more active. The inactivating principle was destroyed by boiling, indicating that the breaking down was of enzymatic nature. UMRATH (1953) also studied the inactivation of substance P by extracts of brain and peripheral nerves. This effect was highly increased after incubating the sterile nerve extracts 19 hours at 38° C.

The inactivation of substance P by proteolytic enzymes in pancreatic juice and smooth muscle is further studied in this paper.

Methods.

Trypsin and Chymotrypsin. Crystalline trypsin and chymotrypsin preparations, prepared at the Chemical Department of Karolinska Institutet and kindly supplied by Professor E. JORPES, were used.

A crystalline trypsin sample, purchased from Worthington Laboratories, New Jersey, USA, labelled TR 21, was also used. This preparate was kindly supplied by Dr. M. ROCHA E SILVA. The activity of the two trypsin preparations was 1 unit per mg and of the chymotrypsin preparation 0.8 units per mg, determined according to WILLSTÄTTER et al. (1926).

Proteolytic principle of intestinal muscle. The routine preparation of the tissue extracts was principally performed according to GULLBRING (1943). The external intestinal muscles of guinea-pig and rabbit were freed from the mucosa, submucosa and serosa, cut into small pieces and ground in a mortar with 3 volumes of acetone and filtered. The dried material was extracted with 3 volumes 0.9 per cent NaCl, filtered and the clear filtrate used immediately.

For the extraction and purification of the proteolytic principle on a larger scale from cow's intestine the following procedure was used. After freeing from adjacent tissue, the muscles were cut into pieces and frozen at — 30° C and minced in a Turmix blender with an equal volume of saline. The homogeneous mass was heated at 50° C on a water bath in 10 min. with continuous stirring. After cooling and filtration one volume of acetone was added to the clear filtrate. After 30 min. the mixture was centrifuged and the precipitate dissolved in smallest possible volume of 2.5 per cent NaCl. The undissolved material was centrifuged off and the supernatant liquid submitted to fractional precipitation with neutral ammonium sulphate. The precipitate obtained with 30 vol. per cent ammonium sulphate was inactive and centrifuged off. To the supernatant was added Am_2SO_4 to 60 vol. per cent. The precipitate now obtained contained all active principle. It was collected by centrifugation, dissolved in distilled water and dialyzed for 48 hours against 2.5 per cent saline at 3—4° C.

The determination of the proteolytic action of the preparations on substance P was performed by biological assay on the isolated guinea-pig ileum. A partly purified preparation of substance P obtained by chromatographic purification (PERNOW, 1953) and containing 200 units per mg was used throughout the experiments. To 100 units (0.5 mg) of substance P in 1 ml water was added 1 ml of the enzyme solution and 1 ml Tyrode solution (pH 8.3). The incubation was performed at 38° C and the activity checked on the guinea-pig ileum every five min. When studying the effect of trypsin and chymotrypsin the gut was previously desensitised towards the enzymes according to the method described by ROCHA E SILVA (1940), which essentially consisted in continuously adding small amounts of trypsin and chymotrypsin to the bath until the response of the gut to the enzyme had ceased.

Some fractions were also tested on the rabbit's blood pressure after having destroyed the enzymatic activity by boiling.

In some experiments the inactivating effect of the proteolytic enzymes on bradykinin was studied parallel to that on substance P. A preparate containing 20 bradykinin units per mg, kindly supplied by Dr. M. ROCHA E SILVA, was used.

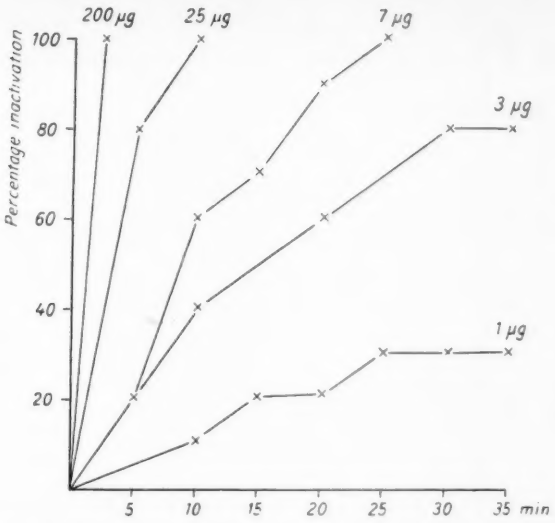

Fig. 1. Inactivation of substance P by crystalline chymotrypsin. 0.5 mg of substance P (100 units) in Tyrode's solution was incubated with 1—200 µg chymotrypsin in 1—35 min. at 38° C.

Results.

Effect of chymotrypsin. Substance P is rapidly destroyed by chymotrypsin. After incubating 100 units of substance P (0.5 mg) with 3 µg chymotrypsin or larger amounts the activity is destroyed within 30 min. (Fig. 1). The percentage inactivation of the smooth muscle stimulating and hypotensive effects of substance P always ran parallel.

Effect of trypsin. Substance P was also inactivated by trypsin but only with amounts far exceeding that of chymotrypsin. Thus the amount of trypsin necessary for 50 per cent inactivation in 15 min. was about 200 times greater than that of chymotrypsin (Figs. 2—3). The same result was obtained with both trypsin preparations used. Bradykinin, which was rapidly inactivated by chymotrypsin, was not influenced by the trypsin preparations. The inactivation of the muscle stimulating and hypotensive effects of substance P by trypsin ran parallel.

Proteolytic principle of intestinal muscle. Intestinal muscle from guinea-pig and rabbit, comprising the circular and the longitudinal

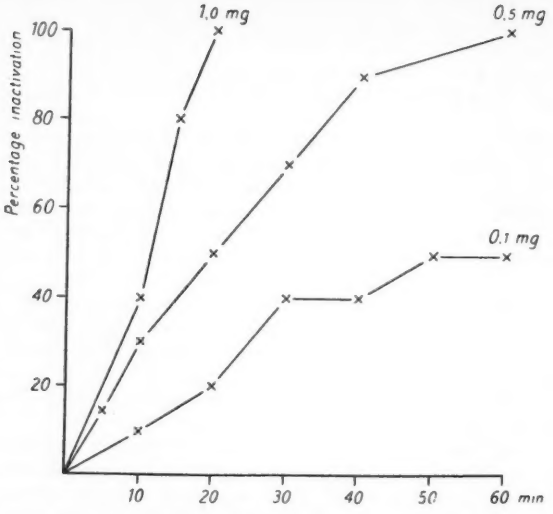

Fig. 2. Inactivation of substance P by crystalline trypsin. 0.5 mg of substance P (100 units) in Tyrode's solution was incubated with 0.1—1 mg trypsin in 1—60 min. at 38° C.

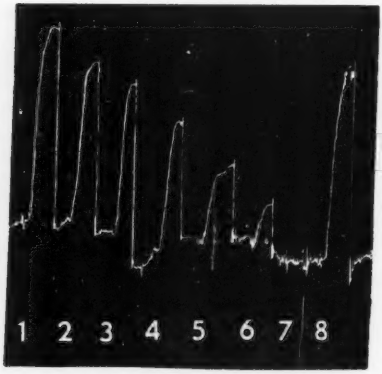

Fig. 3. Isolated guinea-pig ileum, desensitised to trypsin. Bath volume 3 cc. Inactivation of 0.5 mg of substance P (100 units) by 0.5 mg trypsin at 38° C. 1—7 show the effect of the same dose after 1, 5, 10, 20, 30, 40 and 60 min. incubation. 8 = control solution without trypsin after 60 min.

Table 1.

Percentage inactivation of 30 units of substance P in min. by intestinal muscle extracts at pH 7.7 and 38° C.

Expt g wet tissue	Stomach Corpus		Duodenum		Jejunum		Ileum		Colon		Rectum	
	50 %	100 %	50 %	100 %	50 %	100 %	50 %	100 %	50 %	100 %	50 %	100 %
Guinea-pig												
1. 0.13	25	40	15	25	10	20	15	25	25	40	25	45
2. 0.4	15	28	10	16	6	11	10	15	15	25	15	25
3. 0.3			5	7								
4. 0.15			5	10								
Rabbit												
5. 0.6	20	50	15	30	10	25	20	45	35	75	30	65
6. 0.5	15	40	10	20	10	20	15	40	20	50	20	60
7. 1.0	10	20	5	8	5	10	10	20	10	25	20	35

muscle of the stomach, duodenum, jejunum and different parts of the ileum, colon and rectum was studied. The highest proteolytic activity was obtained with extracts from the duodenum and the jejunum. In the ileum, progressively decreasing activity was obtained in an aboral direction. A small inactivating effect was also found in extracts from the colon and rectum (Table 1).

0.5 mg of the purified preparation of the proteolytic principle of intestinal muscles of cow completely inactivated 100 units of substance P (0.5 mg) in 40 min. (Fig. 4). Bradykinin was also rapidly inactivated by this preparation.

The influence of pH on the proteolytic principle of intestinal muscle was studied over a range of pH 4—9. To 1 ml of buffer (0.1 M citric acid/0.2 M Na_2HPO_4 for pH 4—5, M/15 phosphate for pH 6—9) and 2 ml saline was added 50 units of substance P in 1 ml water solution. To each tube was added 0.2 ml of the enzyme solution and the samples were incubated in a water bath at 38° C for 15—60 min. As shown in Fig. 5 the optimal pH for the inactivating process was at pH 7.5—7.8. The same mixture of solution was used as control, the proteolytic enzyme solution having been previously boiled for 10 min. No alteration in the reaction of the gut to this mixture was noted during the experiment. This shows both, that substance P is not influenced by incubation at 38° C at these different pH values, and that the proteolytic principle is destroyed by boiling.

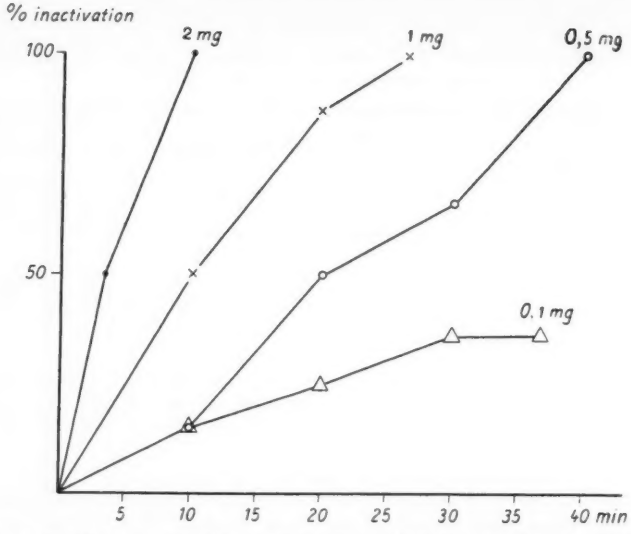

Fig. 4. Inactivation of substance P by the proteolytic principle of intestinal muscle. 0.5 mg of substance P (100 units) in Tyrode's solution was incubated with 0.1—2 mg of the purified enzyme preparation in 10—40 min. at 38° C.

Proteolytic activity of different smooth muscle organs. The inactivating action of smooth muscle from the ureter, urinary bladder and uterus of the rabbit and the guinea-pig on substance P was also studied. The extraction procedure was the same as that described for the intestinal muscle. All extracts possessed the ability of inactivating substance P, although to a lesser degree

Table 2.

Percentage inactivation of 30 units of substance P in min. by different smooth muscle organs at pH 7.7 and 38° C.

Expt g wet tissue	Duodenum		Urinary Bladder		Ureter		Uterus	
	50 %	100 %	50 %	100 %	50 %	100 %	50 %	100 %
Guinea-pig								
1. 0.3	5	7	10	20			10	17
2. 0.15	5	10	15	35			10	20
Rabbit								
1. 0.3	10	20	25	60	70	120	20	50
2. 1.0	5	12	15	50	35	100	10	30

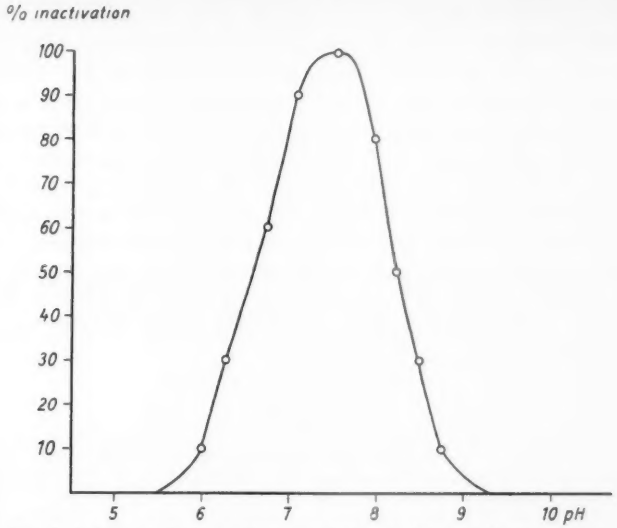

Fig. 5. Influence of pH on the proteolytic principle of intestinal muscle.

than the duodenal muscle (Table 2). The optimal pH for the proteolytic principle was the same for all smooth muscle extracts (7.5—7.8).

Discussion.

Substance P is rapidly destroyed by proteolytic enzymes in pancreatic juice and intestinal muscle. The former is identified as chymotrypsin and, to a much lesser degree, by trypsin. The latter is as yet an unidentified proteolytic enzyme, however, not specific to substance P, since it also inactivates bradykinin. A proteolytic principle with the same pH optimum was also found in the smooth muscle of the ureter, urinary bladder and the uterus. The inactivating power of these extracts was comparable with that of the colon and the rectum, *i. e.* about 60 per cent less than that of the duodenum.

The poor inactivating power of the trypsin preparations compared to the strong and rapid destruction obtained with chymotrypsin is obvious. The difference in effect of the two enzymes is not due to any difference in activity of the preparations as seen from the Willstätter test. It might be suspected that the effect of the trypsin preparates on substance P is not due to the trypsin

per se, but to a possible contamination with chymotrypsin. This seems, however, not to be the case, since bradykinin, which is very sensitive to chymotrypsin, is not affected by the trypsin preparations. Since bradykinin is inactivated by extremely small amounts of chymotrypsin but resistant to trypsin, it offers a sensitive and rapid method to check the purity of trypsin preparations.

The finding by GULLBRING (1943) of a factor in intestinal muscle that inactivates substance P is confirmed. It can be highly purified by fractional precipitation with ammonium sulphate. The distribution of this enzyme in the digestive tract runs parallel to that of substance P. A similar proteolytic factor is, however, also present in smooth muscle organs, where none or very small amounts of substance P are found.

Summary.

1. Substance P is rapidly destroyed by chymotrypsin. 100 units of substance P (0.5 mg) is completely inactivated by 7 μg chymotrypsin in 25 min.

2. Substance P is also inactivated by trypsin. The destroying power of trypsin is, however, about 200 times less than that of chymotrypsin.

3. The strong inactivating power of intestinal muscle on substance P, shown by GULLBRING (1943), has been confirmed. This proteolytic principle has been highly purified by fractional precipitation with ammonium sulphate.

4. A proteolytic factor with the same pH optimum has also been found in other smooth muscle organs, such as the urinary bladder, ureter and uterus of the guinea-pig and the rabbit.

5. Since bradykinin is inactivated by extremely small amounts of chymotrypsin but resistant to trypsin, it offers a sensitive and rapid method for checking the purity of trypsin preparations.

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The Fluorescence of the Serum in Rats with Alloxan Diabetes and Cataract.

II. Spectral Distribution.

By

SVEN ELOV BROLIN.

Received 11 March 1955.

A previous investigation, recently published in this journal (BROLIN 1955) dealt with the problem, whether the amount of fluorescing substances is increased in the serum, when cataract is formed in rats with alloxan diabetes. As this was the case, an explanation was obtained for the raised fluorescence intensity of the eye lens in the course of the formation of a diabetic cataract.

Two alternatives present themselves, when a deposition of fluorescing compounds in the eye lens is considered. The supply may be confined to normally occurring compounds. It is also possible that in addition, abnormal fluorescing metabolites enter the lens to a considerable extent. If such compounds fail to be eliminated in the blood of a diabetic organism, a spectral displacement of the serum fluorescence may take place. A moderate displacement, however, does not necessarily indicate the presence of a new substance since alterations of the spectrum could also be brought about by different distributions of the same compounds between the water and lipid phases of the serum.

The purpose of the present investigation has been to study: *if any spectral displacement of the serum fluorescence is brought about by experimental diabetes giving rise to cataract formation.*

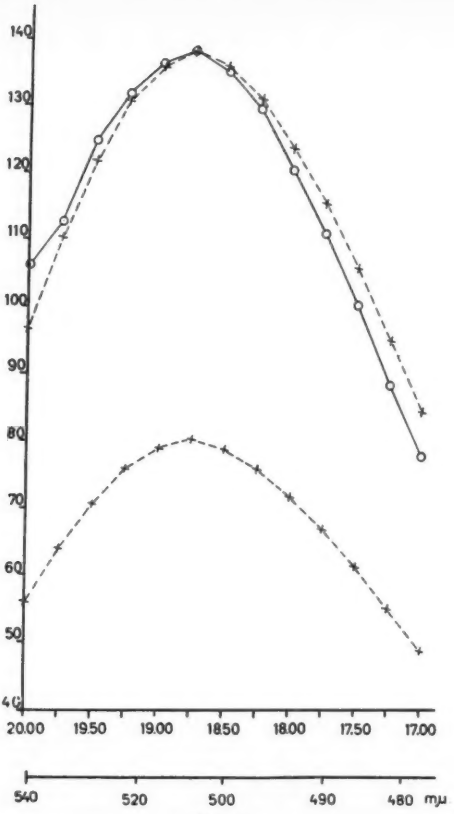

Fig. 1.

Fluorescence spectrograms from serum obtained during the first experimental period. The unbroken line is the mean curve for 11 diabetic rats with cataracts and the lower broken one the mean curve for 20 normal controls. To facilitate comparison the lower curve has been raised to the same height as the other.

The ordinates are galvanometer deflections, and the abscissae the position of the photocell slide with the corresponding wavelength indicated.

Experimental Procedure and Material.

The methods and the material are accounted for in the preceding paper (BROLIN 1955). The experiments were performed during two different periods and double groups were obtained, comprising untreated normal rats, groups of diabetic rats with clear lenses and cataracts and also alloxantreated, nondiabetic groups.

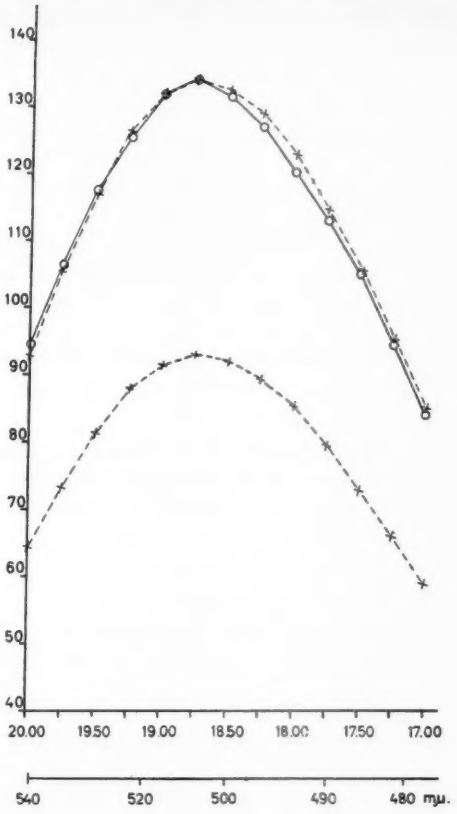

Fig. 2.

Fluorescence spectrograms from serum from the second experimental period, representing 8 diabetic rats with cataracts and 19 controls. The curve convention is the same as in Fig. 1.

Results.

Only slight spectral displacements were observed. In Figs. 1 and 2 a comparison is shown between the tops of the spectrograms obtained from the normal groups and the diabetic groups with cataracts. The maximum values appear to be found at the same wavelength but the width of the measured spectral region was 6 mμ. Thus deviations within this range cannot be excluded.

As seen, a slight shift to the left of the experimental curves was recorded. A considerably weaker and probably unreliable tendency towards the left was shown by the diabetic groups with clear lenses but no such change was found in the nondiabetic groups treated with alloxan. As these curves would scarcely give any further information of special value they have not been presented. Accordingly it remains for us to take the small differences seen in Figs. 1 and 2 in consideration.

The requirements for an estimation of the small differences in question should be present to make statistical calculations convincing. The reproducing ability of the measuring equipment was checked and found to be sufficiently satisfactory. Nevertheless the reliability of the observations are not secured hereby, since different absorption of some wavelengths may result in a biased curve.

To facilitate some calculations on the influence of the absorption on the spectral distribution, the fluorescing light is regarded as emitted from a great number of points, corresponding to excited particles. The symbols used are explained in Table 1.

Table 1.

Definitions of symbols.

E = extinction of the exiting ultraviolet wavelength.

E_{λ_1}, E_{λ_2} = extinction of narrow regions of the fluorescence spectrum (λ_1 and λ_2).

F_{λ_1}, F_{λ_2} and F'_{λ_1}, F'_{λ_2} = intensity of λ_1 and λ_2 at one emitting point and the intensity after passing a length of 0.2 cm.

ΣF_{λ_1} and ΣF_{λ_2} = sum of the intensity of λ_1 and λ_2, being emitted from a large number of points.

I_o, I_{L_1}, I_{L_2} .... I_{L_n} = intensity of the exiting ultraviolet wavelength at the margin of the test tube and after passing the length of L_1, L_2 .... and L_n cm.

L_1, L_2 .... L_n = length of pathway in absorbing solution.

p_1, p_2 = factors by which the intensity of two narrow regions of the fluorescence spectrograms are calculated.

Provided that Lambert Beer's law is applicable:

$$I_{L_i} = I_o \cdot 10^{-E \; L_i};\ i = 1, 2, \ldots . n$$

Now if the fluorescence intensity of different narrow spectral regions is proportional to the intensity of the exciting wavelength the following formula will be obtained.

$$\frac{\Sigma F\lambda_1}{\Sigma F\lambda_2} = \frac{p_1 I_o \cdot 10^{-E \cdot L_1} + \ldots\ldots + p_1 I_o \cdot 10^{-E \cdot L_n}}{p_2 I_o \cdot 10^{-E \cdot L_1} + \ldots\ldots + p_2 I_o \cdot 10^{-E \cdot L_n}} = \frac{p_1}{p_2}.$$

In consequence of this the fluorescence light, emitted from different points, will possess the same spectral distribution even if the intensities are unequal. The distribution may however be changed when the fluorescing light is passing the solution. Now if the absorbing pathway from a single emitting point is as long as the diameter of the test tube (0.2 cm) an unfavourable case is selected and a calculation of the error due to absorption should give a comparatively high value. The following expressions may be used according to Lambert Beer's law.

$$F'\lambda_1 = F\lambda_1 \cdot 10^{-E\lambda_1 \cdot 0.2} \text{ and } F'\lambda_2 = F\lambda_2 \cdot 10^{-E\lambda_2 \cdot 0.2}.$$

Thus the spectral relation $\frac{F\lambda_1}{F\lambda_2}$ is changed by the factor $10^{0.2\,(E\lambda_2 - E\lambda_1)}$.

It is now possible to determine the percentage relationship between the changes caused by the absorption of normal and diabetic serum, whereby only a single emitting point is still considered. Such calculations have been performed when the experiments were carried out in the second period and the results are summarized in Table 2. Because of the fact that the liquid pathways from the emitting points generally are shorter than 0.2 cm, the effect of absorption is still smaller than indicated by the figures of the table. The influence of absorption seems to assert itself in such a way that a deviation to the left of a diabetic fluorescence spectrogram will be counteracted in the first part of the curve and then increased. Even if the source of error caused by absorption seems to be small, it should be considered in the interpretation of the results.

The observed spectral deviations were subjected to statistical calculations in the following way.

All individual spectral curves being obtained in each of the two experimental periods were compared with the mean normal curve from the period in question. After adjustment of the curves to the same height, the surface of the displacement was calculated by the aid of five observations on each side of the top (Table 2). In respect to the normal groups this procedure was only performed to give the standard deviation, the mean being predetermined to zero. The figures of the surface deviations are dependent on the arbitrarily chosen scale unit which does not exert any influence on a probability test. Because of the predetermination of the normal means a routine analysis of variance is not applicable. The following procedure therefore was used. Separate *t*-values corresponding to each experimental period were calculated

Table 2.

Wavelength mμ	540	530	520	510	500	490
Absorption factor per cent	99.6	99.9	100.2	100.0	99.4	98.4

Influence of absorption on the spectral distribution with respect to single emitting points with a liquid pathway of 0.2 cm. It is assumed that spectra with the same distribution are emitted from two corresponding points in serum obtained from normal rats and diabetic rats with cataracts when the experiments were carried out during the second period. In these two groups the mean values of the extinction of different wavelengths have been determined and used for the calculations. Provided that the two mean spectra, which should be recorded, are adjusted to the same height, the percentage figures given above may be calculated. If the intensity values of different wavelengths recorded after passage of normal serum are multiplied by them, the corresponding values after passage of diabetic serum should be obtained. As seen, no artificial contribution to a deviation to the left will be given in the first spectral regions but may subsequently assert itself slightly.

Table 3.

Spectral deviations of the diabetic groups with cataract (D_3, D_4) compared with the normal groups (N_1, N_2).

Groups		Number	Spectral deviation to the left expressed in squares of scale units. Mean	Standard deviation
Series I	N_1	20	0	7.4
	D_3	11	6.4	10.3
Series II	N_2	19	0	8.4
	D_4	8	4.5	7.8

whereby the values 2.00 and 1.30 were obtained. Because the tendency, and also the variation of the series seemed to agree satisfactorily, a common t was determined as the sum of the separate t-values divided by the square root of 2. The common t was 2.33, corresponding to $p < 0.03$.

It is difficult to decide how the probability calculations have been influenced by the errors due to absorption. A slight artificial shift to the left of the right part of the curve may erroneously contribute to a difference. It is also possible that the absorption will increase the variability and in this way form an obstacle to the demonstration of a difference which really exists. Moreover in order to avoid an erroneous evidence, a region of the spectra has been used where no additional deviation to the left is brought about. Such a region is found at 540 mμ since the

absorption due to a hemoglobin peak was little stronger in the diabetic serum which was obtained in both of the experimental periods. Provided that the original spectra were identical, a shift to the right could have appeared in this region but was however not observed (Figs. 1 and 2). The same probability test was applied as above, although the calculations were this time based on one observation of each spectrum and not on computed surfaces. The separate t-values were 1.97 and 0.86. The common t was 2.00 corresponding to $p = 0.05$. The different calculations together seem to support the interpretation that a real deviation to the left of the fluorescence spectrogram is present in the diabetic groups with cataracts.

Discussion.

In an investigation dealing with the formation of experimental diabetic cataract, fluorescence spectra of the eye lens of a few serum samples have been published (Brolin 1954). These serum spectra are not quite reliable because the bleeding technique then used did not prevent hemolysis sufficiently. The most appropriate comparison should consequently comprise the spectrograms of the lens and the recent serum samples. Spectral identity scarcely exists, but the main distribution is common to such an extent that from this point of view a deposition in the lens of fluorescing serum compounds seems to be reasonable.

It is difficult to demonstrate the presence of additional, fluorescing compounds from their influence on the spectrogram. An example is offered by the increased fluorescence of the eye lens observed in rats poisoned with naphthalin (Brolin 1950). A deposition of α-naphthol in the lens was the only reasonable explanation of the increase but it was not possible to prove its presence, because the spectra of the native tissue and α-naphthol were too similar in character. The probable spectral deviation which was found in diabetic groups with cataracts may be caused by additional fluorescing metabolites, but other explanations are also possible. As introductorily mentioned the distribution between water and lipid phases may be altered. The spectral deviation in any case indicates that something more than an increase in concentration seems to occur with regard to fluorescing compounds of the serum, when diabetes gives rise to cataract.

Summary.

The spectral distribution of the serum fluorescence is entirely compatible with the view that fluorescing compounds are deposited in the eye lens, when experimental diabetes gives rise to cataract. In addition to the result made public earlier that the fluorescence of the serum is then raised, a spectral displacement was observed and by statistical calculations found to be probable.

This investigation was supported by a grant from the Swedish Diabetes Foundation. The technical assistances of Mr. R. KIKAS is gratefully acknowledged.

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From the Department of Pharmacology, University of Oslo, Norway.

The Reaction of Striated Mammalian Muscle to Variations of the Parameters of Electrical Stimuli Applied to Nerve and Muscle Investigated with Special Regard to Experimental Technique.

By

EGIL GJONE.

Received 11 March 1955.

The parameters of the directly applied electrical stimuli necessary for threshold responses from muscle are well known and are of fundamental importance for the interpretation of the strength-duration curves. The parameters of direct maximal stimuli, however, do not seem to have been adequately considered in experimental physiology and pharmacology.

Originally the main object of this work was to investigate the influence of variations in the stimuli parameters on directly released muscle responses. In the course of the investigation, however, it became clear that muscle reaction to variations in the parameters of nerve stimuli also required further examination. The results thus obtained form a part of the present paper and provide a basis for the interpretation of those obtained from direct muscle stimulation.

Various experimental techniques have been previously employed for the investigation of muscle response to direct electrical stimulation. It is still under discussion whether or not the striated muscle obeys the "all-or-nothing" law and opinion has varied with time. LUCAS (1905) and PRATT and EISENBERGER (1919) investigated the relation between stimulus and mechanical response

of muscles containing only one single fibre or very few fibres and concluded that the striated muscle obeys the "all-or-nothing" law; ADRIAN (1922) found it highly probable that an "all-or-nothing" relation also existed between stimulus and electrical response.

It was later shown that stimulation with microelectrodes on single muscle fibres gave rise to contractions that were graded according to the strength of the stimulus (GELFAN 1930, 1931, PRATT 1930, BROWN and SICHEL 1930 and ASMUSSEN 1932). GELFAN and BISHOP (1932), however, reported that these graded responses were not accompanied or preceded by action potentials, whereas a characteristic action potential was obtained when maximal contraction of the fibre was elicited. RITCHIE (1932) believes that these graded responses are of the same nature as the local cathodic contractions elicited by a steady current; ADRIAN (1933) rejects them as evidence against the "all-or-nothing" rule.

Stimulation with electrodes extending over the whole length of the fibre has resulted in graded mechanical responses, characterized by the absence of a refractory period (BROWN and SICHEL 1936, SICHEL and PROSSER 1940).

KRUGER (1952) has suggested that the parts of the muscle having "Fibrillenstruktur" obey the "all-or-nothing" law, while those having "Feldernstruktur" give rise to contractions that are dependent on the strength of stimulus.

At present the majority of investigators apparently are agreed that there is no basis for the assumption that the contractile substance of the muscle can only be triggered in an "all-or-nothing" manner (FULTON 1950).

The confusion attached to the principles of muscle response to direct stimulation and the lack of knowledge of the relation between the parameters of the stimuli and the mechanical response necessitate further investigation of this problem.

A systematic investigation of the characteristics of direct stimuli and the mechanical response of skeletal muscle has not been performed, but rather an attempt is made to obtain a reliable basis for experimental technique with respect to direct muscle stimulation.

Most of the previous research into the principles of muscle response to direct stimulation has been performed on frog muscles. Unlike striated mammalian muscle, they most probably react to

nerve stimuli with graded responses in addition to the usual twitch system (KUFFLER and GERARD 1947, KATZ 1949). Skeletal muscles of mammals are now very frequently used in experimental physiology and pharmacology. Striated muscles from rat and rabbit have therefore been chosen for this investigation.

Methods.

A. *Isolated Phrenic Nerve Diaphragm Preparations.*

They were prepared by BÜLBRING's method (1946) and the volume of the bath was 60 ml. Two different electrodes were employed. Both types were right-angled, the shorter arm being furcated to enable fixation of the rib end. The anode was always situated in one of the forks of this arm, whereas the cathode in the one type was placed parallel, 2.5 mm apart, to the anode in the same fork but in the second type lay at the tendinous end.

B. *Rabbit Experiments.*

These experiments were performed on the gastrocnemic and the flexor digitorum longus muscles (cf. NAESS 1949). The preparatory operation was carried out under ether anaesthesia supplemented by local anaesthesia. At least one hour elapsed between the end of the operation and the beginning of the experiment. The animals were either unanaesthetized or under very slight amycal anaesthesia and the hind limb was completely denervated in all experiments.

Nerve stimuli (indirect stimuli) were applied to the severed end of the sciatic nerve. Silver electrodes were used for muscle stimulation before curarization and during complete curarization (direct stimulation). In this article direct stimulation is used to designate muscle stimulation during complete curarization only. The cathode was attached to the tendon which was covered with cotton wool soaked in saline; the anode was placed in the fossa poplitea.

d-Tubocurarine was introduced through a cannula in the jugular vein. Continuous infusion was maintained by means of a specially constructed apparatus. Kymographic registrations after muscle stimulation were made before the infusion of tubocurarine as well as under deep curarization. As the indirectly released high-frequency tetanic contractions are the last to disappear under curarization, their presence may be checked. Controls made before and after each series of direct stimuli confirmed their absence.

A much stronger resistance is offered between the electrodes by the muscle than by the nerve. Two stimulators, that gave "constant current" stimuli with output up to 75 mA within a relatively wide range irrespective of the resistance changes in the external circuit, were therefore employed for muscle stimulation. They transmitted monophasic square wave pulses and the pulse length could be varied stepwise from 25 microsec. up to 1 sec.

A delay box was connected between the two stimulators in the experiments demanding their concurrent use. The delay could be adjusted from 0.1 to 100 msec. and was controlled by means of a cathode ray oscilloscope.

Action potentials were registered from the gastrocnemic and the flexor digitorum longus muscles by means of an Adrian and Bronk's electrode, an A—C amplifier and a double beam oscilloscope. The electrode was fixed securely and a control was made to ensure that there was no deviation from position after prolonged stimulation. Simultaneous registration of the mechanical response was obtained by means of a strain gauge that permitted isometric contraction only.

Altogether, 34 experiments on different phrenic nerve diaphragm preparations of rat and 17 rabbit experiments were performed.

Results.

1. *Mechanical Response.*

a) *Nerve stimulation.*

The muscle response to nerve stimulation is illustrated in fig. 1. The flexor digitorum longus muscle of rabbit was stimulated through the sciatic nerve. Fig. 1 a demonstrates for 5 different pulse lengths the stable maximal plateau obtained with increasing voltage. The muscle responses to three different voltages in an experiment in which the pulse lengths varied stepwise from 0.025 to 10 msec. are registered in fig. 1 b. The relationship between pulse duration and amplitude of contraction is more clearly demonstrated by this figure. The contraction rose above the stable plateau at 0.6 msec. with the highest voltage. In the other experiments, too, this increase took place at short pulse lengths, namely between 0.6 and 1 msec.

b) *Muscle stimulation before curarization.*

Fig. 2 represents the mechanical response of the flexor digitorum longus muscle of rabbit elicited by muscle stimulation before curarization. The variation of the parameters was similar, and the mode of reaction parallel, to that illustrated in fig. 1. With this form of stimulation and stepwise increase of the stimulating current, a constant level was obtained for the shorter pulse lengths. As in the previous experiment, a further increase in the contraction height could only be elicited by increasing the duration of the stimulating impulse. This change in reaction, with consequent augmentation of the muscle response, occurred at 0.6 msec.; in other experiments between 0.6 and 1 msec.

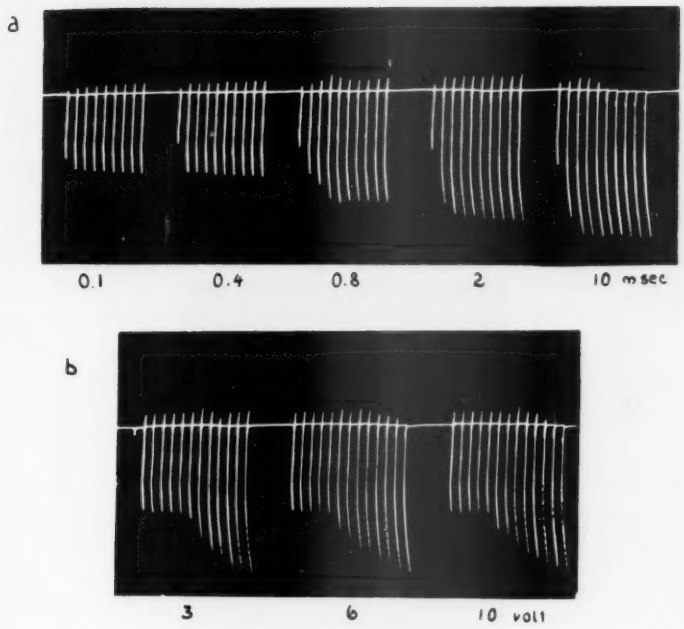

Fig. 1.

Nerve stimulation.
Flexor digitorum longus muscle of rabbit.
a) Voltage increased by steps of 1 volt from 1 to 10 volts in each of the five sections.
Pulse length indicated in msec. on the records.
b) In each of the three sections the pulse length increased through the following steps: 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8 and 10 msec.
Voltage indicated on the records.

c) *Muscle stimulation during complete curarization.*

The contractions elicited by muscle stimulation during complete curarization (direct stimulation) are recorded in fig. 3. Length of impulse and intensity of stimulation were varied as reported above and the results, on the whole, share a similarity with those previously obtained. A maximal plateau was obtained for all the pulse lengths from 0.4 to 3 msec. The main difference between the results obtained before and after curarization lies in the fact that the increase above the plateau took place at a much longer impulse, namely 4 msec. Corresponding results were obtained in all other rabbit experiments with the same muscle. The

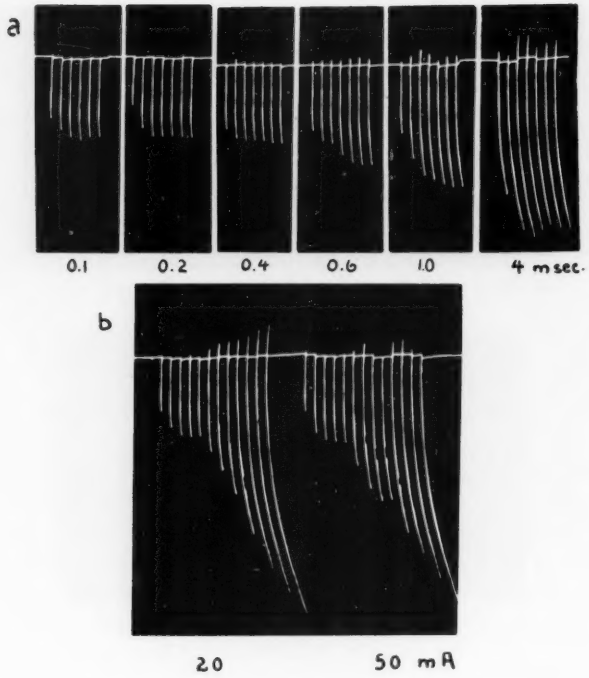

Fig. 2.

Muscle stimulation before curarization.
Flexor digitorum longus muscle of rabbit.

a) In each of the six sections current increased by steps of 10 mA from 10 to 70 mA. Pulse length for each section indicated on the records.

b) With stimulating current of 20 and 50 mA, pulse duration increased through the following steps: 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8 and 10 msec.

maximal plateau, however, was not always as stable as in fig. 3 b, but the first increase above this area was always easily recognized. With maximal current output from the stimulator and increasing pulse lengths, the contractions as a rule first rose above the plateau at 3 or 4 msec.

Increase of the pulse length above 10 msec. caused an augmentation greater that that demonstrated. It is also seen from fig. 3 that the maximal niveau is not so stable as in the previous records. This is probably due to the staircase phenomenon which

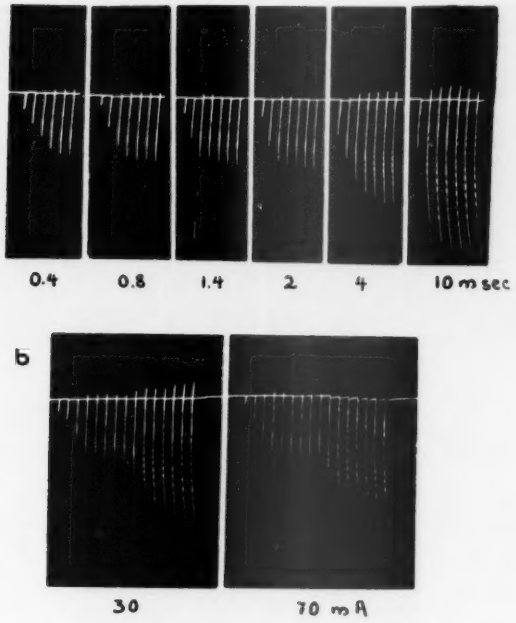

Fig. 3.

Muscle stimulation during complete curarization.
Flexor digitorum longus muscle of rabbit.

a) In each of the six sections current increased by steps of 10 mA from 10 to 70 mA. Pulse length indicated on the records for each section.

b) With currents of 30 and 70 mA in the two series respectively, pulse length increased through the following steps: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 msec.

seemed to be more marked after curarization with this type of interrupted stimulation.

Experiments on isolated phrenic nerve diaphragm preparations of rat gave results that were fundamentally the same but which were not of so clear cut a nature. This was mainly due to a marked gradual decrease in the contractions following direct stimuli of long duration. The results obtained with the two different electrodes were identical and no difference in the mechanical response could be observed. The results obtained with the cathode at the tendon and the anode at the rib end are represented in fig. 4, one of the most successful experiments. The step up from the

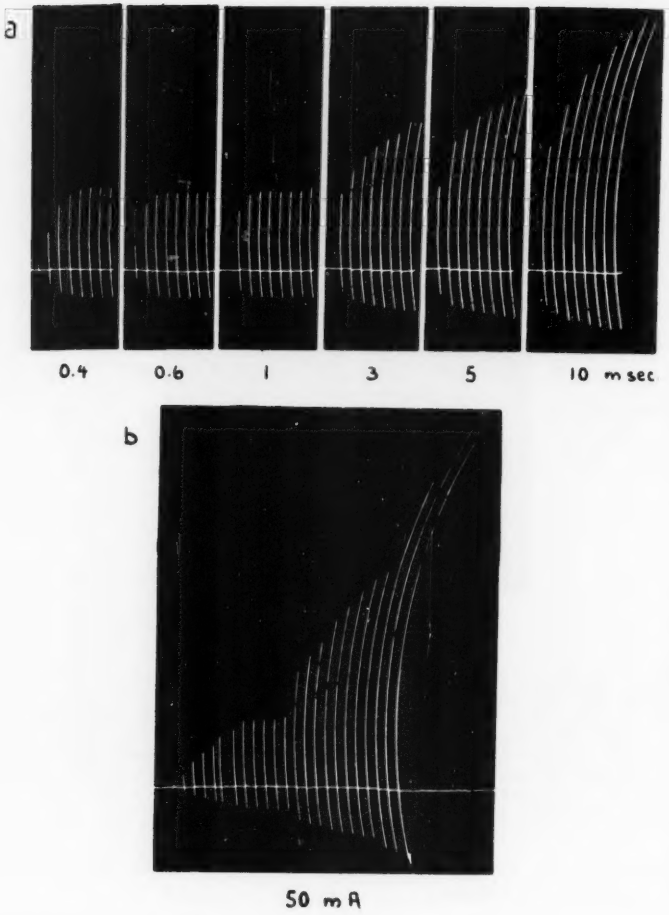

Fig. 4.

Muscle stimulation during complete curarization.
Phrenic nerve diaphragm preparation of rat.
a) Pulse length indicated in msec. in each section. Current increased through the following steps: 10, 20, 30, 40, 50, 60, 70 and 75 mA.
b) Current 50 mA.
Duration of impulses: 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30 and 40 msec.

plateau took place at 3 msec. as was the case in the majority of other experiments.

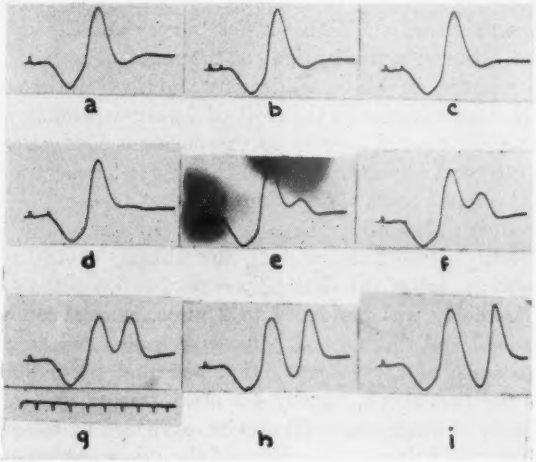

Fig. 5.

Action potentials from the gastrocnemic muscle of rabbit. Nerve stimulation. Pulse length: 0.07 msec.

Strength of stimulus maintained at approximately three times the voltage required for maximal contractions.

First action potential (a) registered after a single shock. Action potentials (b—i) registered following two subsequent stimuli with delay increasing from 0.5 to 3.5 msec. Time in msec.

2. *Electrical Response.*

An investigation of the electrical response to nerve and muscle stimulation was performed to examine the reactions underlying the change of the mechanical response represented by the increase above the plateau.

a) *Nerve stimulation.*

The results following nerve stimulation are illustrated in figs. 5 and 6.

The first action potential (a) in fig. 5 was registered from the gastrocnemic muscle of rabbit after a single maximal shock of short duration to the sciatic nerve. The following potentials (b—i) were read from the same muscle, administered a series of two short maximal shocks with progressively longer intervals between them. The first slight change was noticed on the third potential (c) where the delay was approximately 1 msec.; the first hump-like alteration in the fourth potential (d) at a delay of 1.2 msec.;

an increase of the delay to 1.4 msec. (e) resulted in the addition of a second potential. With further increase of the delay this potential attained normal height at 2.6 msec.

All the records in this figure demonstrate that the time elapsing from the first stimulus to the crest of the corresponding negative spike was 4.2 msec. Contrary to this constant conduction velocity, the second impulse, as far as the shortest delays were concerned, travelled more slowly than the first one.

In records d, e and f with a delay of 1.2, 1.4 and 1.6 msec., respectively, between the stimuli, the distance from the second impulse to the top of the "hump" was read at 5, 4.6 and 4.4 msec. When the delay was prolonged to 2 msec., normal conductivity was restored and the interval between stimulus and crest of the negative spike of the second action potential became equal to that of the first shock, namely 4.2 msec. (record g).

The same phenomenon could also be confirmed by measurement of the interval between the crests of the two negative spikes.

The minimal time distance between the two spikes, even with the shortest delay between the two stimuli, was found to be 1.6 msec.

Identical results were obtained in similar experiments with the flexor digitorum longus muscle.

Fig. 6 represents the muscle action potential from the gastrocnemic muscle, stimulated indirectly through the sciatic nerve. In this experiment single supramaximal (approximately 5 times maximal) shocks of 6 different pulse lengths were employed. The duration of the impulses was read from an adjusted scale on the stimulator. It is seen from the figure that a second action potential was produced by progressively longer impulses. The first noticeable change in the initial potential took place at a pulse length of 0.75 msec. (c); the deformation of the normal potential was clearly visible at a nerve volley of 1 msec. (d); an additional isolated action potential was set up at 1.5 msec. (e).

b) *Direct stimulation.*

Owing to the dominance of the stimulus artefact on the registration, investigation of electrical response to direct stimulation with large currents and long impulses was difficult. An isolating transformer was therefore coupled in between the stimulator and the electrodes. It reduced the stimulating current below that required for maximal contractions but at the same time

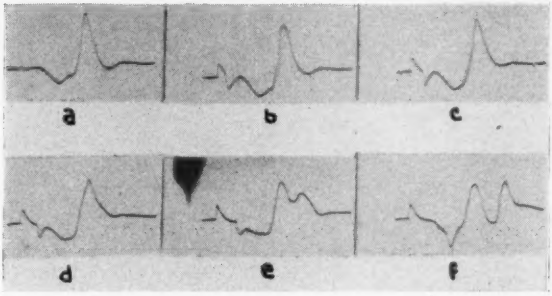

Fig. 6.
Action potentials from the gastrocnemic muscle of rabbit.
Indirect supramaximal stimulation from single shocks.
Pulse duration: a: 0.07, b: 0.5, c: 0.75, d: 1, e: 1.5 and f: 2.5 msec.

allowed registration of reliable action potentials. On account of the weaker currents these results cannot be compared with those obtained with mechanical registration. Nevertheless, there is full agreement between electrical and mechanical registrations. Single action potentials were set up by the shortest impulses; the medium impulses, from 3 to 6 msec. approximately, were accompanied by two action potentials on current make and break respectively. Successive prolongation of the impulse caused an increasing number of action potentials during the current flow. Fig. 7 a records the increase in the number of action potentials for pulse lengths from 2 to 9 msec.

A very short time constant for the amplifier was used for the longer impulses, from 10 msec. to 1 sec., and the consequent deformation of the stimulus artefact permitted satisfactory action potential readings. Fig. 7 b illustrates the repetitive firing during an impulse of 30 msec. duration when a current of 70 mA was employed and the time constant of the amplifier was set at 10 msec.

3) *Refractory Period of Muscle.*

Some experiments were performed to investigate the influence of the pulse duration on the contraction elicited by the break stimulus. Although this definition probably is not quite correct the absolute refractory period was regarded as the time elapsing between the start of the first and the start of the second impulse that caused the first additional augmentation of the contraction.

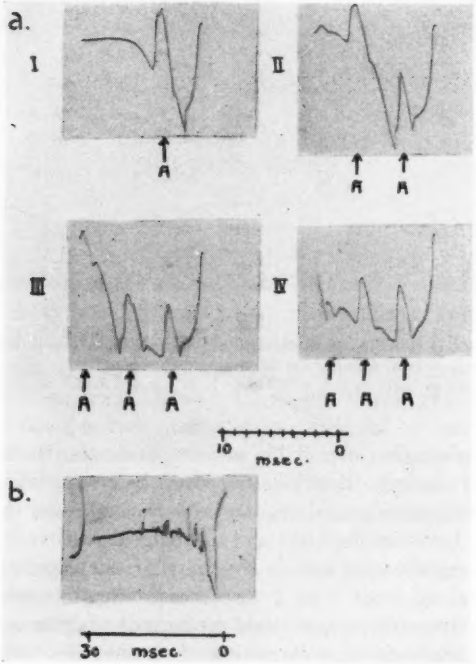

Fig. 7.

Action potentials from the gastrocnemic muscle of rabbit. Direct stimulation.
a) The four tracings refer to impulses of 2, 4, 7 and 9 msec. respectively (to be read from right to left). Time in msec. A indicates action potentials.
b) Pulse length 30 msec., current 70 mA, time constant of amplifier 10 msec.

Two stimulators giving maximal current output of 75 mA plus a delay box were employed in these experiments on fully curarized rabbits. The duration of the first shock ranged from 0.4 to 2 msec. while the second impulse was maintained at 0.4 msec. Consequently the impulses of 0.4 msec. were just maximal and the longer impulses supramaximal.

From experiments of this type it was found that the absolute refractory period following a shock of 0.4 msec. ranged from 1.3 to 1.6 msec. Prolongation of the first impulse caused an increase in the absolute refractory period, *e. g.* with an impulse of 2 msec. duration this period ranged from 2.2 to 2.3 msec.

Discussion.

1) *Nerve Stimulation.*

When triggered by a nerve volley, the muscle fibres obey the "all-or-nothing" rule. Increase in voltage leads rapidly to maximal contractions and further increase of stimulus does not cause any augmentation. These contractions are regarded as single contractions representing the summation of maximal responses of all the muscle fibres present. It is seen from fig. 1 that this statement is valid only for the short impulses. With pulses longer than 0.6 msec., an increase of the contractions is caused by the current break stimulus. With even longer impulses, repetitive firing takes place.

Another experiment in which two successive short nerve volleys were discharged gave parallel results. The first increase in the contractions caused by the second volley occurred at a delay slightly less than 1 msec. The corresponding electrical changes are shown in fig. 5. The first change in the muscle action potential following two subsequent nerve volleys occurred at approximately 1 msec. delay. With one impulse of varying duration the same change also occurred at a pulse length less than 1 msec. (fig. 6). These initial changes in the electrical response represent an additional firing of several fibres.

The fact that an additional contraction occurs at stimuli intervals which are shorter than the absolute refractory period of the muscle requires an explanation. It is obvious that the effect of a second stimulus is primarily dependent on the refractory period of the muscle, as long as that of the nerve is the shorter of the two (0.4—0.5 msec.) (GASSER and GRUNDFEST 1936, GRAHAM and LORENTE DE NÓ 1938).

When the interval between the two successive stimuli is less than 2 msec., the second nerve impulse travels more slowly than the first (fig. 5). The distance between the crests of the negative spikes may be taken as the interval between firing. The minimal value of this interval is seen to be 1.6 msec. and is in good agreement with the value for the absolute refractory period of the muscle in those experiments. On the basis of their experiments on the recovery of nerves, GRAHAM and LORENTE DE NÓ (1938) reported that the longest conduction time immediately after the absolute refractory period was approximately twice the uncon-

ditioned time. ECCLES and O'CONNOR (1939) also found that, at a stimulus interval as short as 0.7 msec., a second nerve volley may excite the motor end plate. Their experiments were performed on soleus muscle of cat and electrodes were applied as close to the muscle as possible.

Thus it may be concluded from the present experiments that the muscle can be triggered by a second nerve impulse also, if the delay is shorter than the absolute refractory period of the muscle. This is made possible by the longer conduction time in the nerve and probably also by a longer conduction time in the motor end plate due to the conditioning shock.

2) *Muscle Stimulation before Curarization.*

It is well known that, unless special precautions are taken, *e. g.* denervation, curarization, special electrodes or use of nerve-free parts of the muscle, pure muscle responses are not obtained. The intramuscular nerve fibres, representing a much more excitable tissue than muscle fibres, are triggered and indirect contractions are elicited. A comparison of figs. 2 and 3 confirms this fact. The decreased excitability after curarization, due to the defunctionalization of the intramuscular nerve fibres, confirms that the nerve fibres are triggered by the made stimulus. The break stimuli also trigger these nerve fibres. This may be deduced from the fact that additional contractions above the plateau have been obtained with impulses of 0.6—2 msec. where the break stimulus occurs within the absolute refractory period of the muscle.

3) *Muscle Stimulation during Complete Curarization.*

The results obtained after complete curarization must therefore provide the basis for the understanding of pure muscle response to direct stimulation. The plateau area and the contractions above this level will be discussed separately.

a) From figs. 3 and 4 it is learned that for the pulse lengths shorter than 3 msec. increase of current may bring the contractions up to a constant maximal level. In addition the contractions cannot be raised above this level even with the use of the strongest currents available. The plateau area must be regarded as representing the maximal contractions possible with this type of stimulation. It is therefore natural to regard the plateau area, comprising the shorter pulselengths, as representing single im-

pulses that elicit the mechanical response, with the action potential as an intermediate link. When this potential sweeps over the muscle membranes present, the whole muscle reacts with the maximal contraction possible. It must be concluded that this reaction obeys the "all-or-nothing" principle as is the case when the muscle cells are stimulated by nerve volleys through the end plates.

Submaximal and maximal contractions, dependent on the intensity of the stimulating current, may be obtained within this area and consequently changes in the irritability and contractility in directly stimulated muscle may be examined.

If the muscle is triggered via the action potential by indirect and by direct stimulation, the resulting maximal contractions should presumably be of equal height. This is not the case. In the same experiments the directly elicited muscle contractions were found to be slightly smaller. This is most probably due to the fact that the stimulus from direct shocks is applied simultaneously to all the muscle membranes present. Therefore the elastic elements of the muscle will restrain the abrupt contraction to a greater extent than they do in the more asynchronous indirectly elicited shortenings.

b) Two possible explanations of the contractions above the plateau for pulses longer than 3 msec. may be discussed.

i) The normal action potential mechanism may be set out of function. In the absence of action potentials, another form of simultaneous depolarization of the whole membrane of the muscle cells may take place, namely one that is longer than the usual action potential and which depends only on the length of the impulse. The graded mechanical responses above the plateau area could thus be explained as a result of depolarization graded according to duration of stimulus. The results from the experiments on rat diaphragm preparations do not support this theory. If such a depolarization should be the cause, presumably the increase in the contractions would only have occurred in the experiments in which the cathode was situated at the tendon. However, identical results were obtained with both types of electrodes.

ii) It therefore seems more probable that the principles relating to nerve stimulation may be applied to direct muscle stimulation. If this were the case, the contractions above the plateau for the medium impulses would presumably be caused by the break stimuli. This theory offers an explanation for the increase up to

from 2—2.5 times the plateau level. For the larger responses following the longer impulses, the occurrence of repetitive firing should be expected.

The registration of action potentials following direct stimulation actually demonstrated that the break of the current for the medium pulse lengths set up a second action potential and that repetitive firing took place when longer impulses were employed.

The reaction of the muscle membrane is thus parallel to the mode of action of the nerve membrane on similar stimulation. There is only one point left requiring further explanation, namely the fact that the initial increase above the plateau takes place at impulses of approximately 3 msec. duration when the absolute refractory period of the muscle after one single short shock has been found to be less (1.3—1.6 msec.). However, the fact that the absolute refractory period is relative to the pulse duration explains fully why the additional response starts at approximately 3 msec. and not at shorter pulses. The increase in the refractory period with these pulses is probably due to changes in the irritability caused by the prolonged current flow through the muscle. Thus when first started, the stepwise increase of the mechanical response, according to the pulselength, is parallel to that occurring after nerve stimulation.

It may be concluded from the present investigation that, under certain experimental conditions, the conducting elements of striated muscle like other excitable tissues may react to direct electrical stimulation in an "all-or-nothing" manner. Maximal contractions to short impulses have been obtained. For longer stimuli, additional responses caused by the break stimuli occur and for even longer impulses repetitive firing takes place. It must be emphasized that these results refer to experiments in which the cathode and the anode have been placed at the same or at opposite ends of the muscle as is commonly done when direct muscle responses are elicited. The muscle reaction to stimulation by more specially constructed electrodes such as fibre length electrodes or microelectrodes applied within the muscle belly has not been investigated.

It must be stressed for experimental work in the field of muscle response to direct stimulation that the upper limit for the time parameter for pure single contractions should always be determined to avoid the influence of double response and thereby unexpected and unreliable results. Many previous experimental

works have not taken the length of the stimulating impulse into proper consideration. Extremely long pulses have been used in the belief that single contractions ensued. Subsequently the registered contraction has been the summation of two or more submaximal responses and has not represented one single maximal contraction.

Summary.

1) The reaction of striated mammalian muscles to electrical stimulation of nerve and muscle has been studied with special reference to the effect of variations in the parameters of the stimulus on the muscular response. Experiments were performed on isolated phrenic nerve diaphragm preparations and on the gastrocnemic and the flexor digitorum longus muscles of rabbit in situ.

2) The absolute refractory period of the flexor digitorum longus muscle following directly applied stimuli of 0.4 msec. has been determined to range from 1.3 to 1.6 msec. Prolongation of the pulse length to 2 msec. caused an increase of the absolute refractory period up to from 2.2 to 2.3 msec.

3) When the interval between two successive short nerve volleys was less than the absolute refractory period of the muscle, an additional electrical and mechanical response could be set up in the muscle by the second volley.

On use of single nerve impulses shorter than the absolute refractory period of the muscle, additional electrical and mechanical responses could also be caused by the break stimuli. This effect has been shown to be a result of the reduced conductivity of the nerve fibres during the first 2 msec. after the conditioning shock.

4) Application of direct electrical stimulation to fully curarized muscle gave the following results:

a) For impulses shorter than 3 msec., single submaximal and maximal contractions were obtained. When the maximal niveau was reached, increase of the stimulating current usually did not cause any further augmentation of the muscle response. It has been concluded that, with this form of direct stimulation, the muscle fibres respond to the stimulus in an "all-or-nothing" manner. It is therefore possible by this method to investigate changes of the irritability as well as the contractility of the muscle itself.

b) With single impulses of 3—6 msec. duration, additional contractions were elicited by the stimulus on current break.

c) Single impulses of even longer duration caused repetitive firing.

Acknowledgement.

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Formation of Bile Acids from Cholesterol in the Rabbit.

Bile Acids and Steroids 29.

By

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The bile acids have been shown to be the main end products of cholesterol metabolism in the dog and the rat (BLOCH, BERG and RITTENBERG 1943, BERGSTRÖM 1952, BYERS and BIGGS 1952, ZABIN and BARKER 1953, STAPLE and GURIN 1954, SIPERSTEIN et al. 1954). We have now studied the conversion of cholesterol to bile acids in the rabbit. It is known that this animal is very sensitive to the administration of cholesterol; the serum cholesterol rises to very high values and the rabbit develops atherosclerosis. Therefore it was of interest to investigate whether the rabbits normally use the conversion of cholesterol to bile acids as an excretory pathway for the cholesterol.

Experimental.

A white albino rabbit weighing about 2 kg was given 0.98 mg cholesterol-4-^{14}C intravenously (490,000 cpm, in aqueous colloidal solution, BERGSTRÖM and NORMAN 1953) The rabbit was returned to its cage and faeces were collected for 2 days. After this period, the gallbladder was removed and a polythene cannula was inserted into the common

bile duct. However, only a few ml of bile fluid were obtained through the fistula and the rabbit died after 12 hours. The gallbladder was extracted with alcohol in a Waring blendor and the activity determined by plating a suitable aliquot (BERGSTRÖM, SJÖVALL and VOLTZ 1953). The fistula bile was made up to a suitable volume with ethanol and an aliquot was plated. The two bile extracts were then combined.

The liver, the small intestine with its contents and the large intestine with its contents were removed and ground in a Waring blendor with ethanol. The organs were then extracted three times consecutively with boiling ethanol for two hours and the extracts were filtered, evaporated in vacuo and weighed. They were then dissolved in a suitable amount of 80 % ethanol and the activity was determined by plating an aliquot giving less than 0.2 mg substance per cm^2 of a copper planchet.

The bile extract and the extracts from the organs mentioned were hydrolyzed in 1 N sodium hydroxide for 5 hours at 110° C in a steel bomb. After acidification, the hydrolysates were extracted three times with ether. The ether was evaporated in vacuo and the residue partitioned between 0.5 N sodium hydroxide and light petroleum to remove the unsaponifiable matter. The aqueous phases were acidified and extracted with ether. After the evaporation of the solvents this ether extract and the light petroleum phases were dissolved in chloroform and suitable aliquots were plated for the determination of radioactivity. The acidic compounds (the ether extracts) were then subjected to a two stage counter-current extraction between equal parts of 70 % ethanol and light petroleum. It was found that a considerable amount of activity appeared in the light petroleum phase. In order to ascertain whether this was due to incomplete removal of unsaponifiable compounds, the residues from these phases were run through a column of Amberlite IRA 400 (BORGSTRÖM 1952). Practically all of the radioactivity was found in the neutral fraction. This illustrates the difficulty in removing small amounts of unsaponifiable matter from large amounts of fatty acids by the procedure employed.

In table 1 the distribution of radioactivity in saponifiable and unsaponifiable compounds is listed. From the foregoing it is evident that the unsaponifiable labelled compounds are contained in the light petroleum phases from the alkali-light petroleum partition and the light petroleum phases from the counter-current extraction procedure. The labelled saponifiable compounds are present in the 70 % ethanol phases from the counter-current distribution.

The acidic compounds were separated by reversed phase partition chromatography as earlier described (BERGSTRÖM and SJÖVALL 1951, SJÖVALL 1953, NORMAN 1953). The following phase systems (NORMAN 1953) were used:

System A: Methanol 180; Water 120; Chloroform 45; Heptane 5.
System C: Methanol 150; Water 150; Sec. octanol 15; Chloroform 15.

The faeces were extracted with ethanol as described for the liver and intestine. The ethanol was evaporated and the residue dissolved in water, acidified with hydrochloric acid and extracted with butanol as

described by NORMAN (1954). The butanol was evaporated in vacuo and the residue was subjected to a two-stage counter-current extraction between 70 % ethanol and light petroleum. The activity was determined as described and the ethanol phases were subjected to reversed phase chromatography.

Table 1.

Per cent of administered activity recovered two days after the intravenous injection of labelled cholesterol into a rabbit.

	Saponifiable per cent	Unsaponifiable per cent	Total per cent
Bile	2.1	0.5	[1]2.6
Small intestine	0.7	2.4	3.1
Liver	4.4	8.2	12.6
Large intestine	5.9	2.6	8.5
Faeces	19.2	[2]2.4	21.6
Total	32.3	16.1	48.4

[1] Gall bladder 1.9 per cent.
Fistula bile 0.7 per cent.
[2] Petrol ether phases (see experimental).

Results.

Table 1 shows the distribution of radioactivity in the rabbit two days after the injection of cholesterol-4-^{14}C. About 30 per cent of the cholesterol had been converted to saponifiable compounds. 60 per cent of these had already been excreted in the faeces. The liver contained the main part of the unsaponifiable compounds recovered in this experiment. The unsaponifiable compounds found in the large intestine and faeces can probably be regarded as excretory products that will not be absorbed, whereas the unsaponifiable material found in liver, bile and small intestine still can be absorbed and metabolized in the tissues of the animal. A bacterial conversion of cholesterol to saponifiable compounds can not be excluded, but evidence presented below does not support this theory. In connection with the distribution of radioactivity in rabbits after injection of ring-labelled cholesterol it may be mentioned that KENDALL et al. (1953) recovered 32 per cent of the administered activity in the liver, lungs, spleen, adrenals and serum of a rabbit 48 hours after the injection.

The saponifiable fractions listed in table 1 were subjected to reversed phase chromatography using phase system A. The distribution of activity in the chromatogram was the same in

bile, liver, small intestine and large intestine. Figs. 1 and 2 show the results for bile and liver. Two main peaks of activity can be found, the largest behaves like desoxycholic acid. The smaller one migrates almost with the solvent front. Since it is known that

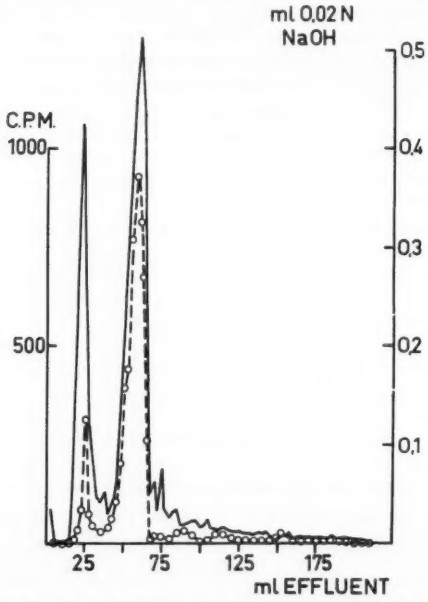

Fig. 1. Chromatography of bile acids from hydrolysed bile (see table 1) 48 hours after the intravenous administration of cholesterol-4-^{14}C. Column 9 g hydrophobic Supercel. Phases: Type A. Titration values: Solid line. Cpm per fraction: Broken line.

rabbit bile contains mainly desoxycholic acid together with a small amount of cholic acid (for references see EKDAHL and SJÖVALL 1955), the front band was suspected to be cholic acid. This band from the bile chromatography was rerun with unlabelled cholic acid in phase system C. The peak of activity coincided with the titration peak. The corresponding band from the liver chromatography was run with phase system C without titration. The activity appeared at the same place as cholic acid. The fractions of this peak were combined and evaporated in vacuo. 50 mg of unlabelled cholic acid was added and the cholic acid was then recrystallized six times from several solvents. The specific activity of the crystals did not change during this process.

The "desoxycholic acid bands" from the bile, liver, small intestine and large intestine were rerun in phase system A for a further purification. The fractions were not titrated, but those containing the radioactivity were combined and evaporated. Six recrystallizations with unlabelled desoxycholic acid did not change the specific activity of the crystals.

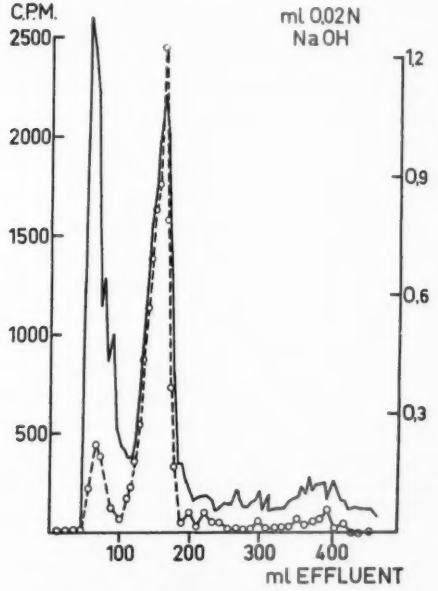

Fig. 2. Chromatography of labelled acidic compounds in the liver 48 hours after the intravenous administration of cholesterol-4 ^{14}C. Column 22.5 g hydrophobic Supercel. Phases: Type A.

Fig. 3 shows the chromatography of one fourth of the faecal acids using phase system A. In addition to the two peaks found in the bile, liver and intestine two new peaks can be seen. These peaks coincide with those expected for desoxycholic acid with one and two hydroxyl groups oxidized to keto groups. They have, however, not yet been identified. Less than 10 per cent of the total activity stayed in the stationary phase where the less polar unsaponifiable compounds would have been found. This indicates that only small amounts of the cholesterol is excreted as unsaponifiable compounds in the faeces.

The front peak of the faeces chromatography was rerun with phase system C. The activity came almost with the front in this system also (that is, was more polar than cholic acid), as was also the case when the corresponding peak from the large intestine was run in the same phase system. No conjugated bile acids could be found in the faeces.

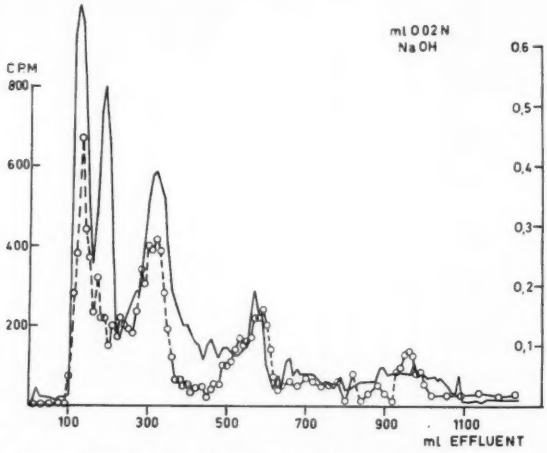

Fig. 3. Chromatography of an aliquot of the labelled faecal compounds excreted during 48 hours following the intravenous administration of cholesterol-4-^{14}C. Column 45 g hydrophobic Supercel. Phases: Type A.

Discussion.

The data obtained in this experiment indicate that the bile acids in the rabbit can be formed from cholesterol. Very little of the administered activity was excreted as unsaponifiable compounds in the faeces.

In a previous paper we have found that when labelled desoxycholic acid was injected into a rabbit with bile fistula the main product excreted in the bile was glycodesoxycholic acid. No free bile acids were found in these experiments (EKDAHL and SJÖVALL 1955). NORMAN has shown that no free bile acids can be found in the bile fluid of bile fistula rats after the intraperitoneal injection of as much as 20 mg free bile acids. In addition there were no free bile acids in the bile when the bile acids had been administered per os and thus been in contact with the intestinal

flora. The labelled bile acids obtained after the injection of labelled cholesterol were found to be totally conjugated in the bile even when they had passed the enterohepatic circulation for several days. Therefore is seems very likely that the bile acids formed from cholesterol in the rabbit have been excreted in the bile conjugated mainly with glycine as they normally occur in the rabbit bile.

When the bile acids have been excreted into the intestine the main portion is reabsorbed and again transported to the liver to be excreted in the bile. The small fraction that is not absorbed comes into the large intestine where the bacteria begin to modify the bile acids. It has been shown in the rat that only minor changes of the bile acids occur before their arrival into the large intestine (SJÖVALL 1955). BERGSTRÖM and NORMAN (1953) have studied the labelled acidic compounds excreted in the faeces after the injection of labelled cholesterol. Whereas in the bile fluid, all bile acids occur conjugated, only a very small part is present in this form in the faeces. GRUBB and NORMAN (1955) have isolated bacteria capable of splitting the peptide bond of the conjugated bile acids and found that many strains of Clostridia and some Enterococci were very active in this respect. NORMAN (1955) has shown that in rats where the intestinal flora had been depressed with antibiotics the faecal bile acids appeared mainly in the conjugated form. Our experiments have shown that in the rabbit too, the peptide bond of the conjugated bile acids had been split and only free bile acids could be found in the faeces.

In our previous paper we found that desoxycholic acid was not converted to cholic acid in the rabbit (EKDAHL and SJÖVALL 1955). The present results have shown that cholesterol gives rise to desoxycholic acid and cholic acid in approximately the same proportion as these acids normally occur in the rabbit bile. A similar condition has been found in the rat where cholesterol forms cholic and chenodesoxycholic acids in the same proportion as these acids normally occur in the rat bile (BERGSTRÖM 1952). Chenodesoxycholic acid, however, does not form cholic acid in the rat whereas desoxycholic acid does (BERGSTRÖM and SJÖVALL 1954, BERGSTRÖM, ROTTENBERG and SJÖVALL 1953). More work has to be done to clear up the reaction steps leading from cholesterol to the different bile acids. It has been suggested that some of the changes in the ring system occur before the cholesterol side chain is degraded into that of the bile acids. This is supported by the

fact that 3α,7α,12α-trihydroxycoprostane is rapidly converted to cholic acid in the rat (BERGSTRÖM at al. 1954, BERGSTRÖM 1955).

As mentioned, the main bile acid in rabbit is desoxycholic acid together with small amounts of cholic acid, both conjugated mainly with glycine. When labelled cholesterol had been administered, however, no cholic acid could be found in the large intestine and several additional labelled compounds could be found in the faeces. This indicates that the bile acids are further modified by the action of intestinal bacteria as has been shown to be the case in the rat (BERGSTRÖM and NORMAN 1953, LINDSTEDT and NORMAN 1955). This is also in accord with SCHMIDT, HUGHES et al. (1942 a, b, 1944) who have been able to demonstrate the oxidation of cholic acid to different keto acids by Alcaligenes faecalis.

Thus the bile acids in the rabbit probably follow the same principal metabolic pathway as the bile acids in the rat. They are excreted as conjugated acids in the bile and enter the entero-hepatic circulation. In each entero-hepatic cycle part of the bile acids are lost into the faeces, the conjugates are split and the bile acids are further modified by the action of intestinal bacteria.

Summary.

The conversion of cholesterol into desoxycholic and cholic acids in a rabbit has been demonstrated. These acids are excreted in the bile mainly as conjugates with glycine. Fractionation of the labelled material excreted in the faeces after the administration of labelled cholesterol has shown that no glycocholic or glycodesoxycholic acid was present. Several unidentified compounds have been formed, presumably by the action of intestinal microorganisms.

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Effect of Large Amounts of Plasma from Desoxygenated Blood on Erythropoiesis.

By

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Received 21 March 1955.

Earlier investigations of the present writer (HIRSJÄRVI 1953) suggest that previously inactive blood gains, in certain circumstances, erythropoietic activity when subjected to lowered atmospheric pressure. The experiments were carried out by injecting small amounts of plasma (1—2 cc plasma/kg body weight) from the exposed blood sample into recipient animals, the same method was also used in earlier investigations on the erythropoietic activity of blood from anoxic animals (BONSDORFF and JALAVISTO 1948, LOESCHKE 1950). The reactions manifested in the recipients were, however, relatively small and inconsistent, and therefore the results could be evaluated only on the basis of statistical comparison with a control material, and yet often remained somewhat uncertain. A natural explanation for this inconsistency of the reaction might be the low concentration of the active substance in the recipient's blood after the injection, which allows the normal fluctuations in the peripheral blood picture as well as, perhaps, the different reactivity of individual recipients to disturb the results. Therefore, a more reliable method for testing the erythropoietic activity were urgently needed. For this purpose, JALAVISTO (1953) used recipient animals, whose haemopoiesis was depressed by small daily injections of blood. This method was, however, handicapped by the formation of isohaemolysins. — ERSLEV (1953) suggested that, in order to get reliable results, large amounts of plasma (50—100 cc/kg body weight) should be injected into the recipients. As such large amounts of plasma must be counted upon to cause haemodilu-

tion, the changes in the peripheral erythrocyte count could not be considered as a criterion for erythropoiesis, and ERSLEV, accordingly, evaluates the results mainly on the basis of reticulocyte reactions. Large injections of plasma from rabbits rendered anaemic by bleeding called forth, in Erslev's material, a very marked reticulocytosis (up to 9 %) in the recipient rabbits. In the erythrocyte count there was a slight initial decrease; a decrease of at least similar magnitude was also observed in a control group of animals, which received equal amounts of normal plasma. In ERSLEV's opinion, the reticulocytosis is strong and constant enough to be relied on as a sole criterion for erythropoietic activity.

The aim of the present experiments is to confirm the erythropoietic activity of plasma from blood subjected to lowered atmospheric pressure in vitro, and on the other hand, to study whether Erslev's technique might successfully replace the older method in investigation of the erythropoietic activity of blood.

Test Subjects and Methods.

As donors as well as recipients untreated male rabbits weighing 1.8—3 kg were used. The animals were kept on a standard diet consisting of oats and swedes, supplemented by spruce twigs or fresh hay; a free supply of water was allowed. Blood to be subjected to low or normal atmospheric pressure was, as a rule, obtained by heart punctures, in a few cases the donor animals were bled from the carotid artery. Heparin was used as anticoagulant. Blood samples were taken simultaneously from 2 rabbits, mixed, and then divided again into 2 parts; one part was exposed to air at a pressure of 10—300 mm Hg, while the other sample was kept at normal atmospheric pressure, and served as a control. The exposure lasted for 1—2 hours. Both the normal and the low pressure sample were centrifuged directly after the exposure, and 10—25 cc of plasma were injected without delaying into normal test rabbits. Each recipient received 3 injections during 3 successive days, the total amount of normal or anoxic plasma injected into one recipient being approximately 50 cc. This was rather less than the amounts recommended by ERSLEV. Preliminary experiments, however, suggested that larger amounts did not cause reticulocyte reactions of a greater magnitude, but would, as it seemed, have a stronger haemodiluting effect.

The reticulocyte, erythrocyte and haemoglobin values of the recipient rabbits were followed before the injections until no major fluctuations were observed, and determined daily during and a few days after the period when the injections were given. Blood for these determinations was obtained by pricking the marginal ear vein and letting

Table

Effect of injection of anoxic and normal plasma

Anoxic plasma						
Date	Recipient	Initial value	Deviation on			
			1st day	2nd day	3rd day	4th day
9.3	1.....	1.9	+ 1.5	+ 3.8	+ 1.4	+ 0.3
23.3	2.....	1.6	+ 1.1	+ 1.5	+ 2.0	+ 1.2
7.4	3.....	2.9	+ 1.0	+ 0.6	+ 3.5	+ 0.4
4.5	5.....	2.4	+ 1.5	+ 1.4	+ 1.1	+ 1.8
12.5	6.....	1.2	+ 1.2	+ 1.0	+ 1.3	+ 0.4
25.5	7.....	0.9	+ 0.6	+ 0.4	+ 0.4	+ 0.3
15.6	3.....	1.7	+ 1.0	+ 1.6	+ 0.5	—
Mean		1.8 ± 0.25	+ 1.2 ± 0.12	+ 1.4 ± 0.34	+ 1.5 ± 0.37	+ 0.7 ± 0.29

Table

Effect of injection of anoxic and normal plasma

Anoxic plasma					
Date	Recipient	Initial value	Deviation on		
			1st day	2nd day	3rd day
9.3	1.....	5.7	— 0.5	— 0.8	— 0.3
23.3	2	4.7	+ 0.1	+ 0.1	+ 0.1
7.4	3.....	5.2	— 0.8	+ 0.3	0.0
4.5	5.....	5.1	+ 0.1	+ 0.2	+ 0.3
12.5	6.....	4.7	0.0	+ 0.1	— 0.2
25.5	7.....	4.9	— 0.5	+ 0.2	— 0.3
	3.....	4.6	+ 0.1	+ 0.1	+ 0.2
	Mean	4.99 ± 0.15	— 0.21 ± 0.16	+ 0.03 ± 0.13	— 0.03 ± 0.10

a drop form. Samples for erythrocyte and haemoglobin determinations were pipetted directly from the drop, and another drop was used to prepare reticulocyte slides. The reticulocytes were stained supravitally with 1 % solution of brilliant cresyl blue, and superstained, after fixing, with Giemsa solution. The counting was performed by means of a Miller ocular, 20 small squares were counted for each determination (BRECHNER and SCHNEIDERMAN 1950).

Results.

The experiments were performed on seven recipient rabbits. All but one received normal as well as anoxic plasma, and thus

I.

on the recipients' reticulocyte percentage.

Normal plasma					
Recipient	Initial value	Deviation on			
		1st day	2nd day	3rd day	4th day
2	2.6	0.0	— 0.3	0.0	— 1.0
1	1.6	+ 0.3	+ 0.6	+ 1.0	+ 0.3
4	1.4	+ 0.9	+ 0.4	+ 0.5	+ 0.7
6	1.6	— 0.4	— 0.3	+ 0.3	— 0.4
5	2.0	+ 0.6	+ 0.5	+ 0.3	+ 0.3
3	2.2	— 0.6	— 0.3	— 0.6	0.0
7	1.5	— 0.6	+ 0.2	— 0.2	—
Mean	1.8 ± 0.17	0.0 ± 0.24	+ 0.1 ± 0.17	+ 0.2 ± 0.19	0.0 ± 0.24

II.

on the recipients' red cell count (Mill./cu.mm).

Normal plasma				
Recipient	Initial value	Deviation on		
		1st day	2nd day	3rd day
2	4.7	0.0	— 0.2	0.0
1	5.1	+ 0.2	— 0.3	— 0.1
4	5.5	— 0.1	—	—
6	4.9	— 0.2	— 0.5	— 0.4
5	5.1	— 0.2	— 0.4	— 0.2
3	4.9	— 0.4	— 0.3	0.0
7	4.9	— 0.7	— 0.6	— 0.4
Mean	5.01 ± 0.09	— 0.20 ± 0.10	— 0.38 ± 0.06	— 0.18 ± 0.06

served as their own controls. The results in regard to erythrocyte and reticulocyte reactions are presented in tables I and II.

A definite difference between the *reticulocyte* values in the experimental and control group is observed (table I): after injection of anoxic plasma there is always an increase of the reticulocyte count, which is pronounced in some cases, while more moderate in others. Normal plasma, on the other hand, caused only occasional, moderate increases in the recipients' reticulocyte percentage. Comparison of the average values given in the bottom line of table I also indicates that there is a significant difference

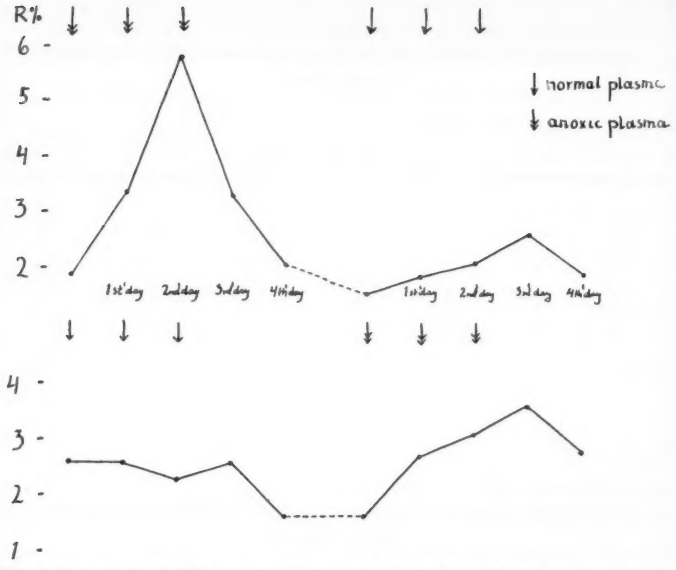

Fig. 1. Reactions of rabbits 1 and 2 to injections of normal and anoxic plasma. Upper curve: rabbit 1, lower curve: rabbit 2.

between the average reticulocyte reactions in the two groups during the three days following the first injection ($P < 0.01$, first day; $0.01 < P < 0.02$, second day; $P = 0.02$, third day). Also, when the average value for the maximal reticulocyte reactions of individual rabbits is calculated in both groups, a statistically significant difference is obtained (anoxic group, average $+ 2.1 \pm 0.38$, normal group, average $\pm 0.4 \pm 0.17$; $P < 0.01$).

In fig. 1 a paired experiment is illustrated. The two rabbits received injections simultaneously, No. 1 had first anoxic plasma, and after a lag of approximately 2 weeks normal plasma, No. 2 vice versa. From the curves the different reticulocyte reaction after anoxic and normal plasma is evident.

In regard to the *erythrocytes*, the difference between the two groups is not as clear (table II). In both groups there usually was a moderate decrease in the erythrocyte count on the day following the first injection. In the anoxic group the values were levelled again on the second day, whereas in the normal group the erythrocyte count was often further decreased on the second

and third day. No statistical difference between the erythrocyte values in the two groups can, however, be discerned except on the 2nd day, where the difference is statistically probable ($0.02 < P < 0.05$).

The haemoglobin values followed approximately the erythrocyte fluctuations. As, however, no significant differences between the haemoglobin deviations in the two groups could be observed, the haemoglobin values are not given here.

Discussion.

The results of the present experiments indicate that injections of large amounts of plasma from blood subjected to low pressure call forth a definite increase in the reticulocyte percentage of the recipients. The effect on erythrocyte count is, in most cases, slightly depressing on the first day, but the values are levelled again earlier than in a control group of animals, which received normal plasma.

When considering the reticulocyte and erythrocyte reactions in individual recipients the question may arise, whether the reticulocytosis in the anoxic group might not be due to an initial decrease of erythrocytes (anaemia — anoxia — stimulated erythropoiesis). Though this possibility may not be totally excluded in some individual cases, it is, however, insufficient to explain why, in the normal group, where the erythrocyte count is equally decreased, there still are no reticulocyte reactions. Thus, the reticulocyte reactions in the anoxic group must be called forth by some other stimulus than anaemization by haemodilution.

The present results are very similar with the results of ERSLEV on plasma from anaemic donors, which has a recognized erythropoietic effect. The fact that the reticulocyte responses in ERSLEV's material were stronger in the average probably depends on the larger amounts of plasma injected. Thus, the present data can be regarded to confirm the theory that blood subjected to lowered atmospheric pressure in vitro gains, in some circumstances, erythropoietic activity.

Another purpose of the present study was to decide, whether ERSLEV's technique would give so consistent results that it could replace other methods in the investigation of erythropoietic substances. The technique is, however, in the writer's opinion handi-

capped by the initial decrease of erythrocytes, which renders the erythrocyte reactions to the active substances very difficult to evaluate. Thus, the reticulocytosis remains the only criterion of erythropoiesis to be relied upon. On the other hand, reticulocyte peaks are known to occur without ensuing erythrocytosis and probably without real stimulation of erythropoiesis (MINOT and CASTLE 1935). Accordingly it seems to the writer that Erslev's technique should not be used alone in the investigation of erythropoietic substances, but completed by some other method, *e. g.* bone marrow examination.

Summary.

The effect of large amounts of plasma from blood subjected to low pressure on rabbits' peripheral blood count was studied. The injections were followed by a definite increase in the recipients' reticulocyte percentage, which was absent in a control group receiving plasma from untreated blood. In the erythrocyte count there was an initial decrease after injections of anoxic as well as untreated plasma, the normal erythrocyte level was, however, regained earlier in the anoxic group. These findings are interpreted as to confirm the theory that initially inactive blood may, in some circumstances, gain erythropoietic activity under exposure to low atmospheric pressure.

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Formation of Myoglobin.

By

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The only iron compound closely related to hemoglobin present in the mammalian organism is myoglobin. It is a low polymerized form of hemoglobin (WARBURG 1948), its molecular weight 17,000 being 1/4 of that of hemoglobin. Its iron content was found by THEORELL (1932), by ROSSI and ARAGONA (1942) and by DRABKIN (1945) to amount to 0.34 p. c. like that of hemoglobin. Recently THEORELL and ÅKESON (1955) crystallized myoglobin from horse heart and muscle. It was composed of three fractions which could be separated by electrophoresis, not however by fractional crystallization. The main fraction obtained and amounting to about 80 % of the total myoglobin content was found to contain 0.297 p. c. iron only.

Text-books of histology do not contain any statement as to the way myoglobin is formed. Studies on the radiation sensitivity, the result of which is to be communicated in this note, suggest, however, that myoglobin is laid down during maturation of "myoglobetic" muscle cells in a similar way as hemoglobin is laid down in the course of maturation of hemopoietic marrow cells.

Experimental.

In each of the first 3 experiments 10 female guinea-pigs with a weight of 450—500 g were injected 5 hours after exposure to 500—1,400 r with 0.25 ml of a physiological solution of sodium citrate containing 3 μg of labelled

iron with 1 microcurie of activity and killed 18 hours later; 10 controls were treated in the same way. Striated muscles weighing abt. 200 g secured from the animals were extracted with cold water and fractionated with ammonium sulfate, as described by THEORELL and ÅKESON. The temperature was kept at + 4° and the pH near 7 during the whole procedure. Colorless protein, hemoglobin and mixtures of hemoglobin and myoglobin were precipitated in a series of steps with increasing saturation. Owing to the great dilution, however, pure myoglobin fractions did not appear until near saturation. The procedure was followed spectrophotometrically by determining the position of the α-band of the CO-ferro compounds, the maximum of which appears at 570 mμ for CO-Hb and at 580 mμ for the corresponding myoglobin compound. After wet combustion of the myoglobin a known aliquote was used for colorimetric iron determination and another was precipitated as sulfide, after its iron content had been increased to 500 μg by adding ferric chloride as a carrier. The precipitate was filtered through a perforated aluminium dish covered by filterpaper (AGNER et al. 1954) and the dish placed under the Geiger counter. A known aliquote of the injection solution was treated in the same way.

In each of three later experiments 12 rats were injected with 0.25 ml of a physiological solution of ammonium citrate containing 2 μg of labelled iron of an activity of 2 microcuries. The first group of rats weighed 300 g each, the second and third group 180 g each.

In all the experiments hemin of hemoglobin was secured from a few ml of blood of the guinea-pigs or rats and the specific activity of the hemin of the hemoglobin determined in a similar way as that of myoglobin iron.

Results.

The results obtained when investigating the effect of exposure to a dose of 43 r per min. for 33 min., thus to 1,400 r, on myoglobin and hemoglobin formation in the guinea-pig are seen in Table 1. Hemoglobin formation is seen to be depressed by irradiation to 1/4 of that of the controls, myoglobin is depressed markedly as well by exposure to radiation but only to 1/2 of that of hemoglobin.

Experiments with rats were carried out both a few hours after irradiation and after the lapse of 23 hours. The results of these experiments are seen in Table 1 as well. The depression of Fe^{59} incorporation into myoglobin of animals exposed to a dose of 500 r is also in these experiments very marked but somewhat less than incorporation into hemoglobin.

The about 5 times slower rate of incorporation into myoglobin than into hemoglobin of the rat indicates a five times longer life-time of myoglobin. This is however possibly an upper

Table 1.

Effect of exposure to X-rays on the incorporation of Fe^{59} into hemoglobin and myoglobin.

	Time in hrs.		Specific activity					
	Between exposure and injection	Between injection and termination of life	Hemoglobin			Myoglobin		
			Control	Irradiated	*Irrad.* Control	Control	Irradiated	*Irrad.* Control
Guinea-pigs	6	18	100	20	0.20	42	18	0.43
			100	22	0.22	22	10	0.45
			100	28	0.28	18	7	0.39
Rats	6	66	100	49.0	0.49	19.8	12.5	0.63
	6	66	100	49.3	0.49	18.4	10.7	0.58
	23	46	100	8.9	0.09	16.1	2.67	0.16
	23	46	100	5.1	0.05	15.0	1.52	0.10

49 p.c. of injected Fe^{59} incorporated in the course of 66 hrs. into hemoglobin.

limit of the ratio of life-times only as the circulating radioiron may reach the muscle cells at a slower rate than those of the marrow. In the guinea-pig we find the rate of formation of labelled hemoglobin to be 3 times that of myoglobin. THEORELL and assoc. (1952) estimated this ratio to be 4, while a value of about 2 follows from the results of HELWIG and GREENBERG (1952).

The daily renewal of the rat hemoglobin [12.8 g per kg body weight (DRABKIN 1950)] being about 1.5 p. c., 58.5 mg of hemoglobin is formed daily in a 300 g rat. The myoglobin content being 0.4 g per kg body weight and its daily renewal amounting to about 0.6 p. c. the daily myoglobin formation works out to be 0.75 mg.

Discussion.

Hemoglobin or myoglobin formed in a medium containing labelled iron is bound to take up Fe^{59}. Incorporation of Fe^{59} into these compounds is thus a measure of their formation rate. A marked depression of Fe^{59} uptake into myoglobin as shown by all our experiments indicates thus a depressed synthesis of this compound in the exposed animal. Myoglobin formation is affected in a similar way as hemoglobin formation, while hemins as cytochrom b or catalase are not (HEVESY and BONNICHSEN 1955).

Irradiation leads not only to a depression of Fe^{59} incorpo-

ration into myoglobin. It is also showing the time-lag of this effect found for the incorporation of Fe^{59} into hemoglobin. As already observed by HENNESSY and HUFF (1950) and later by BELCHER and assoc. (1954) and HEVESY and BONNICHSEN (1955) the full effect of exposure to a few hundred r on the incorporation of Fe^{59} into hemoglobin of the rat manifests itself only after the lapse of about 1 day and the same is the case for the incorporation of Fe^{59} into myoglobin of the rat muscles as seen in Table 1.

The following explanation was suggested for this time-lag (HEVESY and BONNICHSEN 1955). Hemoglobin is known to be laid down in the course of the maturation of erythropoietic marrow-cells. Irradiation interferes with cell division, thus with the formation of such cells, furthermore cells present may be wiped out under the effect of exposure to radiation. The *milieu* in which hemoglobin formation is to be laid down being strongly reduced, hemoglobin formation is bound to be reduced in the exposed animal as well. Shortly after irradiation numerous marrow cells are still present having an incomplete hemoglobin content and these will go on to accumulate hemoglobin, as *hemoglobin formation per se is not radiosensitive.* Only after such cells were wiped out or released into the circulation will the full effect of irradiation on hemoglobin formation manifest itself. In the marrow of the mouse exposed to 400 r the number of nucleated cells was found to be reduced after the lapse of 1 day to only 3 p. c. of its initial value, increasing again after the lapse of 7 days when regeneration of the hemopoietic marrow cells sets in. The time-lag shown of the irradiation effect on hemoglobin formation is also shown for myoglobin formation. While 6 hrs. after exposure incorporation of Fe^{59} into myoglobin is reduced to somewhat less than half, it is reduced to almost 1/10 when investigated 23 hrs. after irradiation. This finding strongly suggests that myoglobin is laid down in myoglobetic muscle cells in the course of their maturation in a similar way as is hemoglobin into erythropoietic marrow cells.

Mitosis is known to be a very rare event in the muscle tissue, however, a very minute mitotic figure would suffice to account for the replacement of myoglobin ending its life-cycle. To replace the hemoglobin of the red corpuscles which sustained physiological death, involving the daily release and subsequent splitting of about 58.5 mg hemoglobin in a 300 g rat, a mitotic figure of 1 in the bone marrow, which makes out about 1 p. c. of body weight, suffices.

The calculation of a mitotic figure of the muscle tissue sufficient to replace the myoglobin molecules which completed their life-cycle leads to the following result.

The myoglobin content of the rat amounts to 1/32 of its hemoglobin content, its muscle tissue is 45 p. c. of its body weight, the life-time of myoglobin at least twice of that of hemoglobin. Thus a mitotic figure amounting to $\frac{1}{32 \times 2 \times 45} = \frac{1}{2{,}880}$ of that of the bone marrow suffices to keep up the myoglobin level in the rat. It is exceedingly difficult to ascertain the presence or absence of such a low mitotic figure (one dividing cell among almost 300,000) in the muscle tissue.

Muscle nuclei are said to divide mainly in an amitotic way. Even an amitotic division has to be preceded by the accumulation of nuclear constituents, and as irradiation is known to interfere with such an accumulation, amitotic arrest would also lead to an interference with myoglobin formation.

Summary.

Incorporation of radioiron into myoglobin of the muscles of guinea-pigs and rats was found to be depressed to a similar extent as into hemoglobin under the effect of exposure to a dose of (500—1,400 r of) roentgen rays, in contrast to hemins, the formation of which is not in close connection with mitotic processes as that of cytochrom b or catalase.

A very pronounced effect of exposure to irradiation on myoglobin formation, as indicated by incorporation of radioiron, manifests itself only after about the lapse of 1 day, which is also the case for hemoglobin formation.

These results are interpreted as indicating the laying down of myoglobin in "myoglobetic" muscle cells in the course of their maturation in a similar way as hemoglobin is laid down in the erythropoietic marrow cells.

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Fatigue of Sustained Tetanic Contractions.

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Before the appearance of MERTON's paper in 1954 it was generally accepted that the transmission in the motor end plate was the first function to fail during artificial indirect stimulation. However, this type of fatigue was not thought to be of any importance for the modification of the naturally induced contraction since application of electrical stimuli to the nerve through the skin was said to elicit powerful contractions when the voluntary produced contraction was fatigued (cf. EVANS 1952).

According to this statement, it should be possible to investigate the limiting capacity of the human motor end plate to transmit impulses by applying supramaximal stimulation with different frequencies and periods of stimulation. Our original intention was to perform an investigation of this kind.

In the course of this series of experiments, which were finished in the latter half of 1953, it was discovered that fatigue during sustained voluntary tetanic contractions was a purely peripheral phenomenon and therefore of the same nature as that during artificial indirect stimulation.

In his far more comprehensive study of this phenomenon, MERTON (1954), who used a similar but more elaborate technique, had come to the same conclusion. MERTON demonstrated, however, that action potentials of almost normal amplitude could be observed in the muscle after a significant decrease in the contractions. He dismissed the previous theory that the block of the neuromuscular transmission was the limiting factor for sustained tetanic contractions, even after artificial stimulation. MERTON

therefore concluded that the biochemistry of the contractile process becomes defective during fatigue and that neuromuscular transmission remains unimpaired.

Our results, mentioned above, could not add any information of importance to those published by MERTON. Some of them, however, were not quite in accordance with his theory and there were also some discrepancies between his results and those previously published by BROWN and BURNS (1949) after almost similar experiments on animals (cf. discussion).

We therefore planned to continue our experiments and supplement those performed on man with animal experiments in the hope of revealing the cause of the discrepancies mentioned above.

Method.

Experiments in Man.

Isotonic contractions of the adductor pollicis longus muscle were elicited by indirect supramaximal stimulation. Natural contractions produced by maximal effort were also recorded. A constant voltage electronic stimulator supplied rectangular impulses of 0.5—1 msec. with different frequencies.

Several technical details of special importance require a closer description.

Supramaximal stimulation is absolutely necessary for an examination of this type. Submaximal stimulation would incur the possibility of a change of stimulation due to minimal movements of the stimulating cathode or to slight changes in the excitability of the nerve during the period of stimulation. Only slightly supramaximal stimulation could, however, be applied, since too strong current causes much pain. The stimulation was increased in the course of 1—2 seconds to a suitable supramaximal strength and maintained in practically all experiments at a constant level throughout stimulation, *i. e.* until the contraction disappeared or reached a stable level.

To ensure that the decline in contraction was not a consequence of a reduced stimulation of the motor nerve, the stimulation was, however, usually increased slightly for a short interval just before it was cut off. As a rule, this procedure had no effect and thus assured that the stimulation was really maintained at a supramaximal level throughout stimulation. Different muscles were examined. Only a few motor nerves, however, are suitable for separate stimulation and often these nerves supply too many and too strong muscles. Long sustained maximal contractions obtained by stimulation of those nerves are usually too painful and cannot be included in investigations of this type. The adductor pollicis muscle was chosen since constant maximal contractions could be obtained without too much discomfort.

The terminal branches of the ulnar nerve were stimulated just below the wrist. An exact localization is necessary and the wet cathode (diameter about 1 cm) must be kept firmly fixed to this point during stimulation, since small movements of the electrode may affect the stimulation, and branches of the median nerve supplying the opponens muscle may be stimulated if the electrode is moved only a short distance towards the central line of the wrist. The anode (about 40 cm^2) was fixed to the ulnar side of the lower part of the forearm.

As a rule, 50—80 volts was sufficient for supramaximal stimulation. Different frequencies of stimulation were used for the orientating investigation, but the standard frequencies employed during the subsequent experiments were 50 and 200 st./sec. 50 st./sec. corresponds to the maximal physiological frequency of stimulation; the higher frequency 200 st./sec. was utilized to test the neuromuscular transmission under very strong unphysiological activity, since it is known from several animal experiments that transmission of impulses in all types of synapses are more rapidly inhibited by use of higher frequencies.

The form and the height of the contractions vary with the load, and the loads were therefore adjusted in relation to the strength of the adductor pollicis muscles. This strength was determined by estimating the maximal weight which could be moved by pure adduction of the thumb. Two thirds of this weight was employed in most of the experiments. The maximal load moved by the adductor pollicis ranged from 5.5 to 7 kg approximately in the different individuals examined in this series, when the load was fixed on the middle part of the thumb over the interphalangeal joint. The movement started when the thumb was maximally abducted, but the lever moved by the thumb and connected to the load was held fast in the starting position to prevent too strong abduction of the thumb and over-stretching of the adductor muscle. Too strong stretching of the muscle would also incur relatively strong reflex activity in the muscle before the period of stimulation and would thus interfere with the starting condition in the muscle. The load was removed from the finger between contractions to allow complete relaxation and good circulation through the muscle during the rest period.

The interval between the different periods of stimulation is of some importance, and the effect of changing this interval was also subject to investigation. 10 minutes, however, was the usual interval between the end of one contraction and the beginning of the next.

Muscular contractions of six normal people were recorded in this series. Most of the investigation was, however, performed on the authors (one male and one female).

The action potentials were recorded by means of an Adrian and Bronk's electrode, an AC-amplifier and an oscilloscope; the simultaneous mechanical response was registered by a strain gauge, a DC-amplifier and the other beam of the cathode ray tube.

The time base of the oscilloscope could be blocked for arbitrarily chosen intervals and single potentials, even after tetanic contractions,

could therefore, be recorded once a second. This procedure allowed a more exact analysis of the quantitative and qualitative changes in potential. Action potentials elicited by single shocks were recorded before and after the tetanic contractions to ensure that no displacement of the electrode occurred.

Experiments in Rabbit.

The preparatory operation was performed during combined ether-procaine anaesthesia. To exclude any effect of the anaesthesia on the results, $1^1/_2$—$2^1/_2$ hours were allowed to elapse before the investigation was begun. The hind limb was denervated and electrodes for indirect stimulation were placed on the peripheral end of the severed sciatic nerve. Mechanical registration of isotonic contractions was made from the flexor digitorum longus muscle and experimental conditions were maintained as similar as possible to those in experiments with the adductor pollicis in man. The muscle was loaded with a weight approximately two thirds of the maximal force produced by the muscle on tetanic stimulation (0.6 to 1.0 kg) and supported in a resting position corresponding to that obtained by a stretch of 0.2 to 0.3 kg on the muscle. The muscle was allowed complete relaxation for the 10 minutes elapsing between contractions.

Electrodes for direct stimulation were placed on the isolated tendon and in fossa poplitea. The action potentials were recorded from the soleus and gastrocnemius muscles in two different ways, namely by means of an Adrian and Bronk's electrode and by means of a monopolar electrode in the belly of the muscle and an indifferent electrode on the tendon. The electrical arrangement was otherwise similar to that used in the experiments with man.

Supramaximal stimulation with rectangular impulses of 0.1 msec. was used for indirect stimulation. Until recently it was uncertain whether the muscle cells themselves react in an all-or-nothing manner or give responses graded according to the strength of stimulation. GJONE (1955) in our laboratory has shown that mammalian skeletal muscle obeys the "all-or-nothing" rule. Maximal responses may therefore be obtained and supramaximal stimulation can also be used for direct stimulation after curarization. Stimuli with a duration of 2 msec. and a strength of 30 mamp., which have been shown to be definitely supramaximal for the flexor digitorum longus muscle, were therefore used in this investigation.

Results.

1. *Mechanical Recordings from Man.*

Fig. 1 demonstrates the constancy of the response of the adductor pollicis muscle to indirect stimulation. When the muscle has to contract against a constant, heavy load, the contractions dis-

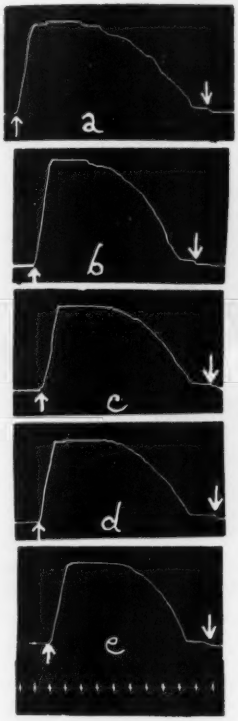

Fig. 1. Isotonic contractions of the adductor pollicis muscle in man, produced by indirect supramaximal stimulation with a frequency of 50 per second (for details cf. methods). Time in 5 seconds. Interval between contractions: 10 minutes. Arrows mark beginning and end of stimulation.

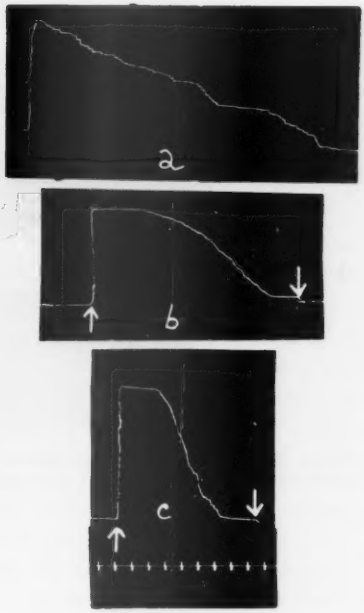

Fig. 2. Isotonic contractions of the adductor pollicis muscle in man. a) voluntary contraction produced by maximal effort; b) and c) contractions produced by indirect supramaximal stimulation with frequencies of 50 and 200 per second respectively. Time in 5 seconds. Interval between contractions: 10 minutes.

appear almost completely in the course of 40 seconds. The first contraction lasts somewhat longer than the proceeding ones.

Our results indicate that the ability to maintain tetanic contractions of this kind varies from time to time in the same subject, but a definite statement on this matter requires further research. Only a few individuals, two of whom have been comprehensively examined, are included in this investigation. There

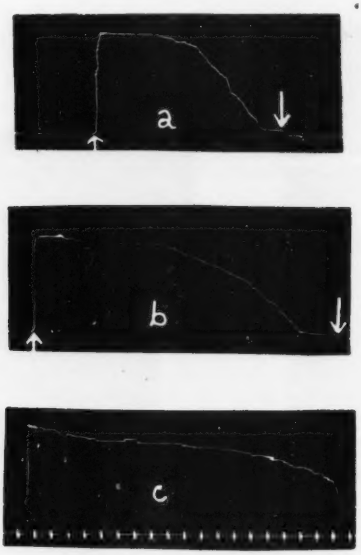

Fig. 3. Similar to fig. 2, but contractions in reverse order *i. e.* a) and b) elicited indirectly by 200 and 50 per sec. respectively. c) elicited by maximal voluntary effort.

seemed to be a significant individual variation in the duration of the contraction, even though the load was adjusted to the strength of the different subjects.

The first contraction in fig. 2 was obtained by maximal voluntary effort, the second one by supramaximal indirect stimulation with 50 st./sec., and the last one with 200 st./sec. The contractions obtained by artificial stimulation are by no means greater or of longer duration than the voluntary one. As a rule, the contractions produced by 200 st./sec. have a somewhat larger amplitude than those induced by 50 st./sec.

To ensure that the sequence of contractions played no significant rôle, the order was reversed (fig. 3). Three contractions induced by stimulation with 200 st./sec. followed by one elicited by 50 st./sec. are recorded in fig. 4.

Figs. 2, 3 and 4 demonstrate that the duration of the responses, and consequently to a certain extent the work performed by the muscle, depend upon the frequency of stimulation.

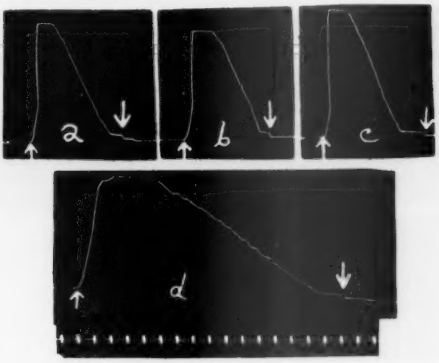

Fig. 4. a), b) and c) produced by 200 per second. d) produced by 50 per second. Otherwise similar to fig. 1.

Fig. 5 illustrates one of the experiments performed to investigate the importance of the interval between contractions. When the interval — the time from the end of one contraction to the beginning of the next — is only two minutes, the recovery period is obviously too short to maintain constant responses. Other experiments have revealed that the response remains constant with intervals of ten minutes, and practically constant with intervals of five minutes. An exception must be made for the first contraction, which even with an interval of 10 minutes is slightly longer than the other ones.

Fig. 6 records experiments in which indirect stimulation was superposed upon maximal voluntary contractions. It was expected that this procedure would have the ability to stabilize or reinforce the declining voluntary contraction. A very short and scarcely noticeable stabilization was obtained, but the principal result, rather astonishingly, was the arrest of the contractions.

2. *Mechanical Recordings from Rabbit.*

Fig. 7 represents results typical of a series of investigations performed in a manner almost similar to those already described. The contractions obtained by indirect stimulation were fairly similar to those obtained by indirect stimulation in man. As a rule, similar contractions were elicited by supramaximal direct stimulation and indirect stimulation, but it happened that the

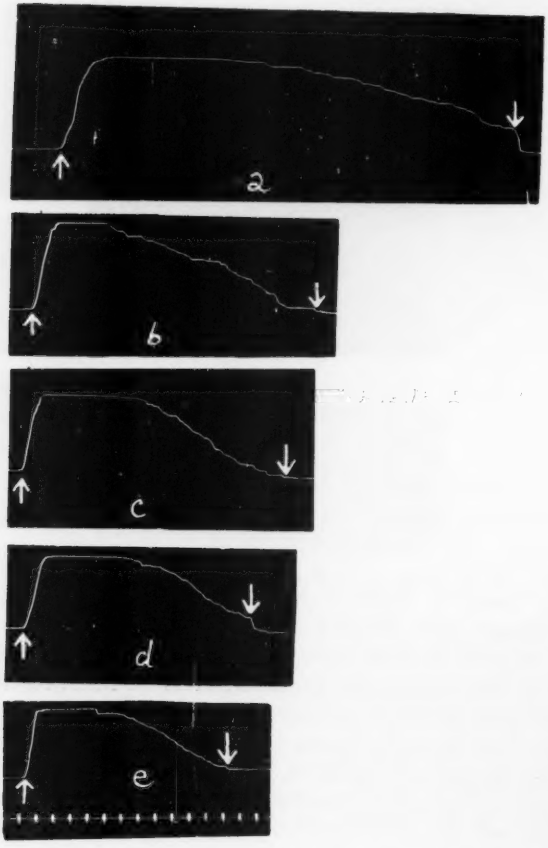

Fig. 5. Intervals between contractions: 2 minutes only. Otherwise similar to fig. 1.

first directly produced contraction had a slightly longer duration than the other ones.

Action Potentials from Rabbit.

Action potentials from the gastrocnemius and soleus muscles were recorded in a series of experiments intended as a basis for similar experiments in man which are very painful.

All experiments gave the same results — a reduction in action

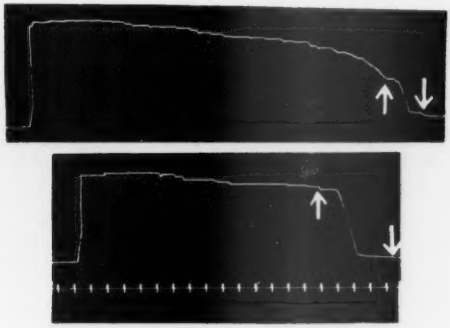

Fig. 6. Voluntary isotonic contractions of the adductor pollicis muscle of man. At ↓, superposition of indirect stimulation with a frequency of 50 per second. At ↑, termination of stimulation. Time in 5 seconds.

potential that was significantly greater than that in mechanical response. The potentials were also longer during the reduction in amplitude (cf. fig. 8).

No great difference was obtained between the two muscles under investigation, but the contraction of the soleus muscle was a little more prolonged.

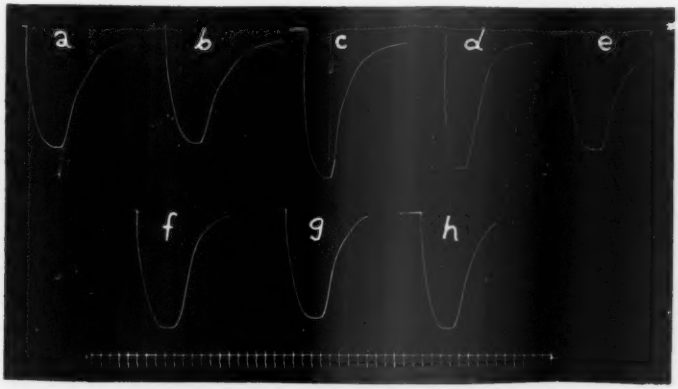

Fig. 7. Isotonic contractions of the flexor digitorum longus muscle of rabbit (for details cf. methods).

Upper series: supramaximal indirect stimulation a), b) and e) frequency of 50 per second. c) and d) frequency of 200 per second.

Lower series: supramaximal direct stimulation with a frequency of 50 per second. Time in 5 seconds. Interval between contractions: 10 minutes.

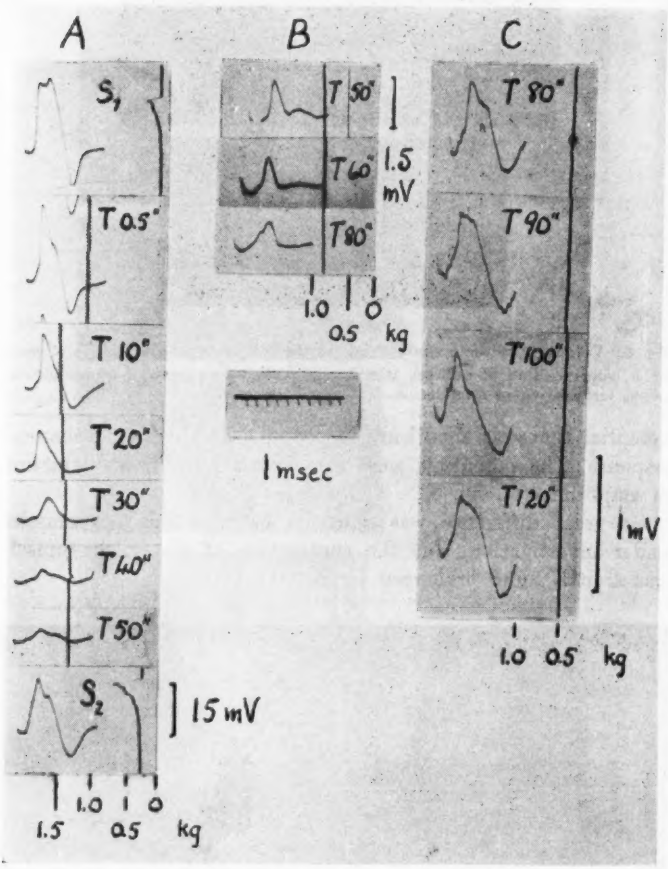

Fig. 8. Action potentials and isometric contractions from the isolated gastrocnemius muscle of rabbit.

S_1: single contraction immediately before tetanic contraction.
S_2: 30 seconds after the termination of the contraction.
T: registrations from tetanic contraction.

Time after beginning of the tetanic stimulation given in seconds. Series A, B and C are from the same experiment, but represent different contractions produced by indirect stimulation with 50 stimuli per second. The three series are recorded at different intervals of time after the beginning of the contraction and with different amplification of the action potentials. Time base circuit blocked by a special arrangement allowing registration of one single action potential every second during tetanic contraction.

Action Potentials from Man.

Due to the pain incurred, only a few experiments of this type were performed. The reduction in action potential and mechanical response of the adductor pollicis muscle was more in proportion. Otherwise the results agree with those obtained from rabbit experiments (cf. fig. 9).

Displacement of the electrode could not be responsible for the changes in potential. Action potentials were in our experiments always reduced to the same extent, even if the position of the electrodes was changed between recordings, and we were never able to obtain stable potentials during this type of contraction.

Discussion.

Our results agree with the statement made by MERTON (1954), that the fatigue during sustained tetanic contractions is a purely peripheral phenomenon. According to his investigation, it is obvious that their decline is due to suppression of the circulation during contraction. MERTON found no significant decrease in action potential, and therefore concluded that muscular fatigue is due to an exhaustion of some biochemical process in the muscle cell. BROWN and BURNS (1949), who performed somewhat similar experiments in cat, also stated that neuromuscular block could not in any way be responsible for the fall in tension. These authors found, however, that neuromuscular block occurs regularly at frequencies of nerve stimulation within the physiological range, but they were of the opinion that this block was never absolute in the single motor end plate, and that nerve stimuli were transformed to a lower frequency during the process of fatigue. The stimuli, thus passing through the motor end plate during partial neuromuscular block, would, according to their view, produce a far stronger reaction than usually. BROWN and BURNS propose that two different mechanisms, previously discovered, are responsible for this increase in activity.

These authors found as a rule that direct stimulation of the muscle does not produce stronger or more prolonged effect than indirect stimulation. Due to a rapid decrease of the excitability developing from fatigue, the strength of stimulation in these experiments was not always believed to be supramaximal. We

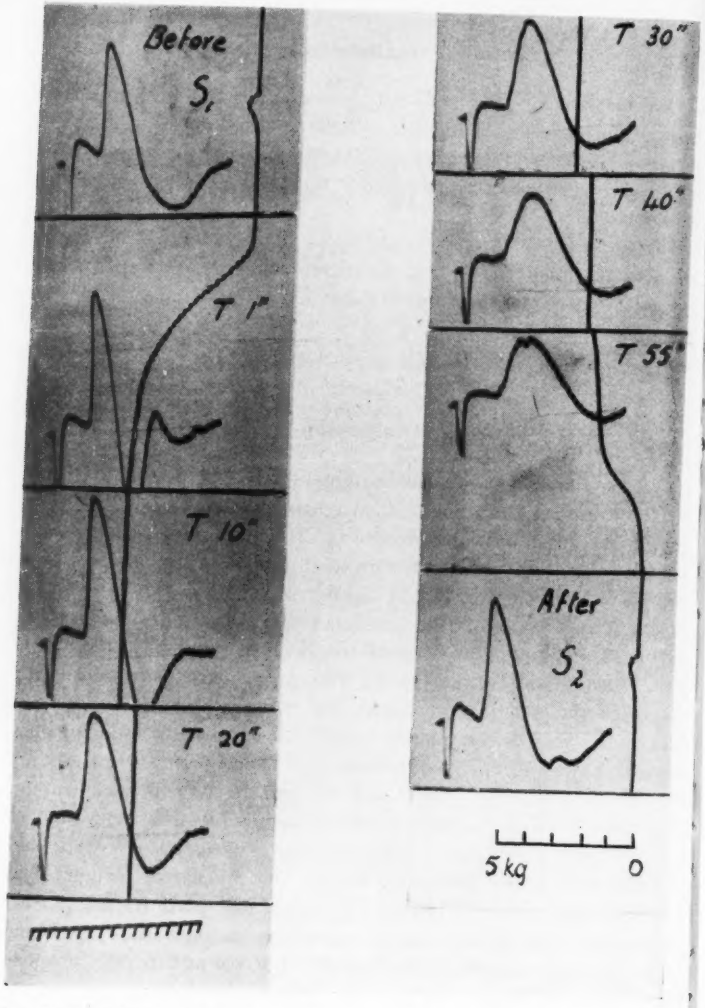

Fig. 9. Experiment similar to that represented by fig. 9 with the adductor pollicis muscle of man. Corresponding method of registration from one contraction only, marking and frequency of stimulation, as in fig. 8.

used decidedly supramaximal stimulation (cf. methods) and came to the same conclusion. As a control the strength of stimulation was increased from 30 to 80 mamp. when the contraction was

about to disappear, without any effect. These facts seem to exclude the possibility that the transmission in the motor end plate plays any significant rôle in the fall of tension.

This conclusion does not seem to be compatible with the fact demonstrated by BROWN and BURNS, that the contraction may be restored to normal amplitude during the fall in tension under indirect stimulation by exchanging indirect stimulation with direct for short intervals. A neuromuscular block must exist and the muscle cells cannot be exhausted when indirect stimulation is used.

BROWN and BURNS also stated that the degree of neuromuscular block (estimated as response frequency of the muscle fibres) is determined solely by the number of volleys which have passed down the nerve. This relationship does not hold for frequencies below 40 per sec., since the authors could not demonstrate any block even after a long train of impulses.

The results of BROWN and BURNS do not agree with MERTON's conclusion, that an exhaustion of the muscle cells themselves is the only cause of fatigue, but the latter makes no comment on this discrepancy.

BROWN and BURNS have recorded electrical activity in the soleus muscle of cat during long sustained tetanic activity. They describe two action potentials with different latencies but mention only changes in the potential with the longer latency. The behaviour of the action potentials is described for a relatively short period of the contraction only. However, these authors draw the conclusion that the electrical records from their experiments support the view that the muscle fibres do not follow the rhythm of the nerve impulses during prolonged stimulation of the motor nerves.

As obvious from our recordings we always found a significant reduction in action potential. The decrease in electrical activity was parallel to the reduction in mechanical response in the experiments with adductor muscle of man, but was proportionally far greater in rabbit. The strong reduction in action potential in the rabbit experiments was very striking, and with the relatively good maintenance of the tetanic contractions in mind one could imagine that some components of the muscle have smaller action potentials and that this type of muscle cell is more fitted for long sustained tetanic (static) contractions.

KRÜGER (1952) has stated that two types of muscle cell with

very different properties exist in mammalian muscles as well as in frog muscle, but KUFFLER et al. (1951) has discarded this possibility.

It is very difficult to explain the divergent results obtained by MERTON and us with respect to the changes in action potential. MERTON used superficial skin electrodes as standard method, but controlled his result with intramuscular electrodes. He is of the opinion that the mechanical response of the muscle cells is more sensitive to the asphyxia produced by suppression of the circulation than the electrical activity of the muscle cell and the neuromuscular transmission. Direct experiments with rat phrenic nerve diaphragm preparation have, however, shown that neuromuscular transmission is blocked by anoxemia before the mechanical response to direct stimulation ceases (ELLIS and BECKETT 1954), but the difference in sensitivity is not very pronounced at normal temperature (37° C).

It is apparent from the experiments performed by BROWN and BURNS that stimuli with frequencies below 40 per second will not be blocked in the neuromuscular junction of all cats. Individual variations seem to exist in cat, and this may also be the case in man. Neither MERTON nor we have investigated the action potentials in more than a few individuals, and it is therefore still impossible to make any definite statement on the existence and significance of individual variations, but the different results point to this possibility.

Even if the action potentials are suppressed during the activity, it is impossible to localize the process involved in the motor end plate proper. Changes may either take place in the terminal nerve fibres or in the sole plate or it may be that the ability of the muscle membrane to propagate impulses is impaired. It is, however, more natural to assume that the motor end plate proper, which has always been considered the limiting factor for transmission of impulses in the peripheral motor system, is also responsible for this depression.

Our logical conclusion is therefore as follows: Circulation is suppressed during strong tetanic contraction and the asphyxia thereby produced affects the processes involved in the response to indirect stimulation. The reduction in action potential suggests that the neuromuscular junction in certain individuals may be the limiting factor when indirect stimulation is used. Consequently the muscle cells are protected and may respond with

strong contractions when direct stimulation of short duration is applied during depression of transmission (cf. results of BROWN and BURNS). The sensitivity of the muscle cell proper and that of the neuromuscular junction to the asphyxia are, however, very similar (cf. ELLIS and BECKETT) and it is therefore almost impossible to produce a more sustained contraction by continuous direct than by indirect stimulation.

Our results from rabbit indicate that the contraction is more easily restored when fatigued by indirect than by direct stimulation. The recovery process takes place more easily and is more reproducible in the motor end plate than in the muscle cell itself. The neuromuscular depression therefore seems to serve a salutary purpose during strong sustained tetanic contractions *i. e.* it protects the processes involved in the contraction of the muscle cell against exhaustion.

The abolition of the contraction in man subsequent to superposition of artificial stimulation during maximal voluntary contractions may also be explained in terms of a strengthening of the muscular block. As obvious from our results, contractions produced by higher frequencies are more readily depressed than those elicited by lower frequencies. This may be explained by the well known tendency of the motor end plate to produce Wedensky inhibition (*e. g.* during curarization). It is therefore quite natural that the function of the neuromuscular transmission working at its maximum breaks down when artificial stimulation is added to maximal natural stimulation.

In 1895 JOLLY introduced faradic stimulation of individual muscles to demonstrate myasthenic reactions. In a review of different diagnostic methods for myasthenia gravis, OSSERMANN and KAPLAN (1952) make the following statement on Jolly's method: "A normal muscle will remain contracted for a minimum period of five minutes whereas a myasthenic muscle will become exhausted in less than five minutes (usually one or two minutes)." As obvious from our results this statement does not hold for heavier loads, and Jolly's method must therefore be performed with far easier loads than those used in our experiments.

In myasthenia gravis, muscular contraction will be exhausted even with the easiest loads and suppression of the circulation plays no rôle in the reduction of the muscular force in these patients.

According to our results and those of BOYD and BURNS, the

block of the motor end plate taking place during sustained tetanic contraction in normal animals and man is therefore not related to the depression of the neuromuscular transmission in myasthenia gravis.

Summary.

The purpose of this investigation has been to examine the mechanism behind the process of fatigue during sustained tetanic contractions.

Mechanical responses and action potentials have been recorded from the adductor pollicis muscle in man and from the flexor digitorum longus, gastrocnemius and soleus muscles in rabbit.

The following results were obtained:

1. Our results from man support the view of MERTON (1954) that the process of fatigue developing during a sustained tetanic contraction is a purely peripheral phenomenon.
2. It is impossible to produce a stronger or more prolonged tetanic contraction by indirect supramaximal stimulation than by maximal voluntary effort.
3. Superposition of indirect stimulation during maximal voluntary effort shortens the duration of the contraction.
4. We found in contrast to MERTON that the action potentials always decrease during the reduction of the mechanical response.

The results are discussed in relation to previous investigations in this field, especially to those of BROWN and BURNS (1949) and MERTON (1954).

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The Effects of Muscle Extracts on the Oxidation of Palmitic Acid by Liver Slices and Homogenates.[1]

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In 1950, LUNDSGAARD published observations on a "factor" present in fresh blood which increased the oxygen consumption by perfused cat livers. Although attempts to identify this factor were negative, it was ascertained that a perfused limb produced the substance or substances necessary for maximal oxygen consumption by the perfused liver. Since the technique of organ perfusion was awkward as a system for exploring this possibly complex interrelationship, attempts were made to simplify the assay methods by testing the effects of muscle extracts on various metabolic functions of liver slices and homogenates. In the course of these experiments, it was observed that isotonic extracts of rat muscle exerted marked effects on both the oxygen consumption and on the rate of conversion of labeled palmitic acid to CO_2 and ketone bodies. The data to be presented give the basis for this conclusion, and in addition demonstrate a partial purification of muscle extracts employed.

[1] Part of these results have been reported in a preliminary form.

[2] This work was done while the author was a post-doctoral USPHS Fellow. The present address is the Department of Metabolic and Endocrine Research, Michael Reese Hospital, Chicago, Illinois.

Experimental Methods and Procedures.

Incubation of Liver Preparations.

In the experiments performed with liver homogenates, the system employed was that used by WENNER, et al. (1953) with modifications cited in the appropriate tables. In all experiments where palmitic acid was used as substrate, no $MgSO_4$ was added to the media to avoid precipitation. It was essential that the liver be well chilled before homogenizing in the POTTER-ELVEHJEM apparatus (1936) in phosphate-buffered isotonic KCl. In other experiments, liver slices were cut free-hand while working in a cold room. These slices were also incubated in phosphate-buffered isotonic KCl solution, but it was not necessary to have exogenous diphosphopyridine nucleotide or cytochrome c in the media to achieve optimal endogenous metabolic rates. In all manometric experiments using homogenates, the gas phase was air, while in manometric experiments using slices, the gas phase was oxygen. Concentrated alkali (0.2 cc 10 % KOH) was present in the center well to absorb CO_2. At the end of an hour's incubation in Warburg vessels at 37° C., 0.2 cc 3 N H_2SO_4 was added from a side arm to stop metabolic processes and to facilitate the complete collection of CO_2. The liver donor animals were male albino rats,[1] weighing between 150—250 grams, which had been allowed to eat *ad libitum* from a diet[2] which permitted good growth.

Preparation of Muscle Extracts.

Crude muscle extracts were prepared from the chilled, pooled skeletal muscles of white rats[1] sacrificed by a blow on the head. One volume of muscle (usually about 100 grams from four rats) was homogenized in a Waring Blendor for 60—90 seconds in three volumes of isotonic KCl buffered with 0.01 M phosphates to pH 7.4 or 7.6. The homogenate was centrifuged. In earlier experiments, the supernatant was centrifuged at high speed (ca. 25,000 × g) to get a particle free solution. After it was determined that a protein-free extract retained full activity, the centrifugation was performed at low speeds (2,000 rpm), and the supernatant was treated chemically to precipitate all proteins. In preliminary experiments, the impression was obtained that muscle extracts were richer in biological activity if the animal donors were starved rather than fed. Consequently, all rats used as a source for muscle extract were fasted for approximately five days prior to sacrifice. Previous to this, they had been maintained on the same *ad libitum* diet that the liver donor animals received.

[1] These rats were kindly donated by the Løvens Kemiske Fabrik, Copenhagen, Denmark.

[2] The diet consisted of milk and animal food checkers from Løvens Kemiske Fabrik.

Proteins were precipitated from crude muscle extracts in a variety of ways, and the final method chosen for routine preparation was that of STEINER et al. (1932), who employed $Fe_2(SO_4)_3$ and $BaCO_3$. The supernatant after this type of precipitation has been shown to be essentially free of the ions of the precipitating substances, and was therefore convenient for subsequent bioassay and further purification. Since there is not nearly as much protein in such muscle extracts as in plasma, less $Fe_2(SO_4)_3$ was used to accomplish a complete removal of proteins. For every 35 cc muscle extract, 2 cc of a 17 % solution of the ferric salt and 2.0 g of $BaCO_3$ were added with vigorous stirring. After centrifugation and filtration, 0.1 cc of a saturated solution of Na_2SO_4 was added to precipitate any barium which remained in solution. Aliquots of this filtrate were then added to the incubation media in the majority of cases to be reported. Between experiments, the muscle extract filtrate was kept frozen. In experiments where plasma was tested for the presence of "factor(s)" larger amounts of precipitating substances were necessary to obtain complete removal of proteins.

Fractionation Procedures for Partial Purification of Muscle Factors.

The protein-free muscle extract solutions retained activity after being shaken with an excess of acid alumina, and also after being passed through the Permutit anion exchange resin "deacidite". The factor(s) were heat stable at the time intervals tested (ten minutes in boiling water). In contrast, the muscle extract lost its metabolic effects if it were percolated through columns of the washed cation exchange resins, Permutit "Zeocarb 216" or Dowex 50, both previously charged with HCl and washed with water until neutral. Preliminary experiments using dilute ammonia as an eluant of the Zeocarb column failed to release any metabolically active fraction. However, elution with 1 N HCl of the Dowex 50 column previously loaded with the protein-free muscle extract released the active factor(s). The material used in experiments 72 and 73, cited in Table III, was derived from fractions of the HCl eluant, after the HCl had been removed by Ag_2CO_3 precipitation and the resulting solution had been taken to dryness *in vacuo*. The metabolically active fractions of the eluant had only non-specific end-absorption in ultra-violet, as measured in the Beckman spectrophotometer, and were free of phosphates, indicating the probable absence of adenine-containing compounds. Further fractionation has not been completed yet. Details of fractionation will be included in a subsequent publication.

Analytical Procedures.

In all experiments reported, the oxygen consumption by liver slices and homogenates was measured by conventional manometric methods. In some experiments, the conversion of various C^{14}-labeled substrates

to CO_2 and ketones was also measured. The substrate predominantly employed was C^{14}-carboxyl labeled palmitic acid, but some separate observations were made using C^{14}-carboxyl labeled sodium acetate, and C^{14}-glucose labeled uniformly. All these substrates were purchased from the Radiochemical Center, Nr. Didcot, Berks., England. When one of these substrates was present in the incubating media, each vessel contained one microcurie of activity. The stock palmitic acid (0.1 mc weighing ca. 28 mg) was dissolved in 2.0 ml 0.01 M KOH, and aliquots were removed from the clear, heated solution. These aliquots were diluted with warm isotonic KCl solution, and appropriate amounts were added to Warburg vessels after all other ingredients except liver had been introduced. The other radioactive substrates were handled in a similar fashion with the exception that no KOH or heat was needed to make the glucose or Na-acetate go into solution.

When a labeled substrate was present, the total C^{14} activity in respiratory CO_2 was measured at the end of the experimental period. The procedure was a standard one, such as that employed by WENNER, et al. in which the filter paper and residual KOH are quantitatively transferred to an appropriate test tube with a known quantity of carrier Na_2CO_3 in KOH solution. Three $BaCO_3$ plates were then made from aliquots of the solution, and total counts per minute per flask were determined. Data are reported after correcting the $BaCO_3$ plates of 2.7 cm^2 for self absorption to a standard plate weighing 16.0 mg. The counting was done with a thin-end window Geiger-Mueller tube and an automatic scaler in duplicate or triplicate for each plate, the time being recorded for the registration of 4,096 counts.

In addition, total counts per minute in total ketone bodies per vessel were determined. Acetoacetic acid and β-hydroxybutyric acid were converted to acetone and the latter distilled by the procedure of WEICHSELBAUM and SOMOGYI (1941). The acetone was precipitated as the Denigés' complex and was counted directly as in the procedure of MEDES et al. (1953). Corrections for self absorption were made on the basis that the Denigés' precipitate absorbed approximately the same as $BaCO_3$ plates. Data are reported in total counts per minute per 16 mg plate of area 2.7 cm^2. All results were then multiplied by two on the assumption that the activities in carboxyl and carbonyl carbons of the ketone bodies derived from carboxyl-labeled palmitic acid were the same. Prior to decarboxylation and distillation of ketone bodies, 1.5 mg acetone was added as carrier to each sample. Results were obtained in duplicate for nearly all vessels.

Results are expressed as total counts per minute per mg of dry liver weight which appeared per hour of incubation in the products from a given substrate. In all cases the same amount of initial activity (59,000 cpm) was present in each vessel for each substrate tested. The data are also expressed as a ratio obtained by dividing the total counts going to ketones by total counts going to CO_2, since it was found that the addition of muscle extract markedly affected this ratio. The oxygen consumption is expressed by the conventional Q_{O_2}, namely microliters of oxygen consumed per mg dry tissue weight per hour.

Results.

Effects of Muscle Extract on the Metabolism of Liver Homogenates and Slices.

In preliminary experiments, it was observed that crude, particle-free muscle extracts raised the oxygen consumption of liver homogenates in the presence of different substrates in the media. Table I has data from some selected experiments demonstrating this phenomenon. It can be seen that the augmentation by muscle

Table I.

Effects of Rat Muscle Extracts on the Oxygen Consumption of Rat Liver Homogenates.

Experiment Number	Substrate	Q_{O_2} of Control Vessels	Q_{O_2} After Addition of Muscle-Extract	Percentage Change
45*	Acetate	11.5	19.2	+ 67
46*	»	14.5	21.0	+ 45
47*	»	16.0	21.4	+ 34
41*	Palmitate	9.2	13.9	+ 51
44*	»	8.4	10.2	+ 21
48*	»	9.9	20.7	+ 109
77	»	12.4	27.4	+ 121
62	Butyrate	17.3	23.5	+ 36
64	»	13.5	22.8	+ 69
65	»	14.2	21.3	+ 50
66	»	14.0	17.9	+ 28
69	»	14.1	23.6	+ 67
70	»	14.7	26.2	+ 78

Legend to Table I.

General incubation procedures are described under *Methods* in the text. Each flask had a total fluid volume of 2.6 cc, having 0.14 M KCl; 0.015 M Na_2HPO_4 plus KH_2PO_4 adjusted to pH 7.4 when acetate and butyrate were the substrates, and pH 7.6 when palmitate was the substrate; 0.003 M $MgSO_4$ when acetate and butyrate were the substrates, and none when palmitate was the substrate; 0.001 M sodium adenosine triphosphate (Pabst); 0.0015 M diphosphopridine nucleotide (C. F. Boehringer & Sons); and 3×10^{-5} M sodium fumarate. The substrate concentrations were 1.7×10^{-3} M sodium acetate; 5.2×10^{-4} M potassium palmitate; and 8.4×10^{-3} M sodium butyrate, respectively. The gas phase was air; the temperature was maintained at 37° C.; and the shaking rate was approximately 120 oscillations per minute. Each flask had exact aliquots of liver homogenates, usually weighing approximately 15 mg when dried at 100° C. The same liver was used for all vessels in any single day's experiments. The amount of muscle extract present was either 0.5 or 1.0 cc, while the fluid volume in all vessels was equal. In the experiments marked with an asterisk, crude muscle extract was added. In all other experiments recorded, protein-free muscle extracts were used.

extract varied considerably, from 21 to 121 per cent, while a few earlier experiments not recorded in Table I failed to indicate any rise in oxygen consumption. The reasons for this large variability include several possibilities. In earlier experiments, the absolute Q_{O_2} values by liver homogenates were lower than in later ones, resulting primarily from a lack of thorough chilling of livers before homogenizing. Also, a protein-rich muscle extract was used in earlier experiments while a protein-free muscle extract was employed after it was observed that the same effect could be achieved after precipitation of proteins. The presence of these proteins may have influenced oxygen consumption. Finally, many variations of an unknown nature undoubtedly existed throughout these studies, such as the changing metabolic states of the liver or muscle donor rats. At any rate, the inconsistent results noted in Table I made this procedure an unreliable assay tool for examination of various muscle portions. Also, this procedure alone gave no information about which broad classes of substrates were being oxidized at a more rapid rate during the period of generalized increased oxygen consumption. It therefore was obviously necessary to obtain more rigorous criteria to aid both the purification of "muscle factors" and in the exploration of possible sites of action.

Consequently, experiments were performed using C^{14}-labeled substrates to study the effects of muscle extract on their conversion by liver. The initial experiments, recorded in Table II, with liver homogenates suggested that total activity from glucose appearing in CO_2 plus ketones was approximately the same in both control and muscle extract-treated liver homogenates. When acetate was the primary substrate, there was less activity in CO_2 plus ketones. In marked contrast, when palmitate was the substrate under consideration, there was far more activity in CO_2 plus ketones after the addition of muscle extract. With all three substrates, relatively more C^{14} appeared in ketones than in CO_2 after addition of muscle extract, independent of the total activities in these two products. This can be seen in Table II, where representative data of the first few experiments are reported. For each substrate, the ratio $\frac{\text{total cpm in ketones}}{\text{total cpm in } CO_2}$ was greater in the muscle extract-treated vessels than in the control ones. When palmitic acid was used as the substrate for liver

Table II.

Effects of Muscle Extract on the Oxidation of C^{14}-Labeled Substrates by Liver Homogenates.

Substrate	Control Vessels		Muscle Extract Added to Vessels	
	Total CPM in CO_2 and Ketones	Ratio of $\frac{\text{Total CPM in Ketones}}{\text{Total CPM in } CO_2}$	Total CPM in CO_2 and Ketones	Ratio[1]
Glucose..	20.0	0.15	18.0	0.48
Acetate..	218.2	0.78	46.3	1.70
Palmitate	454.0	0.49	777.0	2.62

[1] Ratio: $\frac{\text{Total CPM in Ketones}}{\text{Total CPM in } CO_2}$.

Legend to Table II.

All results are expressed as total activity per mg dry liver weight per hour incubation time at 37° C. Each figure is the average value for two separate experiments. The constituents of the basic media were the same as in Table I. Total counts per minute initially present in each vessel were 59,000. Special conditions for each substrate: glucose: 84×10^{-3} M; acetate: 1.7×10^{-3} M; palmitate: 5.2×10^{-4} M. All other constituents were the same as in Table I. All these experimental values are probably too low, since the oxygen consumption was only about half of that achieved in later experiments, probably because the livers had not been sufficiently chilled before homogenization. However, the relative values are valid.

slices, similar results were obtained after the addition of muscle extract. The results depicted in Table III show that in each separate experiment the total amount of C^{14} appearing in CO_2 and ketones from palmitic acid was greater after the addition of muscle extract to liver than in the control slices, even though the absolute activities varied greatly from animal to animal. The most consistent result was the high *relative* amount of activity appearing in ketones after the addition of muscle extracts, independent of both the absolute level of conversion to CO_2 and ketones, and the substrate employed. More C^{14} appeared in ketones than in CO_2 after addition of muscle extract when palmitate, acetate, or glucose was the labeled substrate being metabolized. As with liver homogenates (Table I), the data of Table III also clearly indicate an increased Q_{O_2} of liver slices after addition of muscle extracts. However, both the absolute oxygen consumption and the absolute amount of activity appearing in CO_2 and ketone bodies per mg dry liver weight were higher with homogenates than with slices.

Table III.

Effects of Muscle Extract on the Oxidation of C^{14}-Carboxyl Labeled Palmitic Acid by Rat Liver Slices.

Experiment No.	Control Vessels			Muscle-Extract Added to Vessels		
	Q_{O_2}	Total CPM in CO_2 plus Ketones	Ratio[1]	Q_{O_2}	Total CPM in CO_2 plus Ketones	Ratio[1]
50	8.1	75	0.47	14.0	199	2.38
53	10.9	61	0.46	14.2	372	1.54
66	9.2	117	0.55	19.2	574	1.61
69	8.0	54	0.22	23.8	321	3.45
70	7.3	35	0.58	11.7	83	2.63
71	8.0	54	0.66	14.6	271	3.56
72	8.3	64	0.99	12.1	220	3.45
73[2]	8.0	115	1.35	10.0	184	3.03

[1] Ratio: $\frac{\text{Total CPM in Ketones}}{\text{Total CPM in } CO_2}$.

[2] Experiment 73 vessels had no fumarate present in the incubation media.

Legend to Table III.

All activities are expressed as total counts per minute per mg dry liver weight per hour incubation time, with approximately 30 mg dry weight present in each flask. Each vessel had a total fluid volume of 4.6 cc, containing 0.14 M KCl; 0.015 M Na_2HPO_4 and KH_2PO_4 buffered to pH 7.6; 0.001 M ATP; 9.8×10^{-5} M sodium fumarate (except in experiment 73); and 2.6×10^{-4} M potassium palmitate containing 59,000 counts per minute. Other conditions were as described in text under *Methods*. Special conditions: protein free muscle extract, prepared as in *Methods*, present in 1 cc amounts in following experiments: 50, 53, 66, 69, 70 and 71; partially purified muscle extract eluted from Dowex 50 column as described in *Methods* was present in 0.5 cc amounts in experiments 72 and 73. See text.

Presence of "Factor(s)" in Cat Plasma.

Since it appeared well established from the above data that extracts of muscle exerted definite action upon some phases of liver metabolism, it was of importance to determine if any similarly acting materials were present in the circulating plasma. Blood was obtained from the intact cat; from a perfused liver at varying time intervals after beginning the perfusion; and from a perfused hind limb preparation that was exercised by electrically induced stimulation during part of the experimental period.

Blood from the intact ether-anesthetized cat was obtained from the carotid artery. In a liver perfusion experiment, performed in the manner described by KRUHØFFER and MUNTZ (1954), samples of blood were removed from the posterior vena cava

Table IV.

Effects of Protein-free Serum from Cats on the Conversion of C^{14}-Carboxyl Labeled Palmitic Acid by Rat Liver Slices.

Experiment No.	Control Vessels			Vessels with Serum Added			
	Q_{O_2}	Total CPM in CO_2 plus Ketones	Ratio[1]	Q_{O_2}	Total CPM in CO_2 plus Ketones	Ratio[1]	Source of Serum
76	9.5	83.1	0.71	10.0	41.3	0.99	Intact Cat
78	8.5	32.6	1.23	7.5	46.9	0.91	
66	9.2	117.0	0.56	8.5	85.0	0.50	Posterior vena cava immediately before liver perfusion
69	8.0	54.0	0.22	10.2	49.3	0.30	
66	9.2	117.0	0.56	9.3	68.5	0.44	Venous return 1 hour after beginning liver perfusion
69	8.0	54.0	0.22	8.3	55.6	0.27	
66	9.2	117.0	0.56	13.1	95.3	0.78	Venous return 15 min. after beginning limb perfusion
69	8.0	54.0	0.22	13.7	95.6	0.77	
78	8.5	32.6	1.23	11.9	52.2	1.24	
66	9.2	117.0	0.56	17.7	237.0	2.00	Venous return 5 hours after beginning limb perfusion
69	8.0	54.0	0.22	16.8	115.5	1.30	
78	8.5	32.6	1.23	11.3	80.3	1.55	

[1] Ratio: $\frac{\text{Total CPM in Ketones}}{\text{Total CPM in } CO_2}$.

Legend to Table IV.

The same constituents and conditions were used as in Table III. Serum samples were prepared as described in the text under *Results*. The dilution of protein-free serum samples was 1 : 1, and one cc aliquots were added to each test vessel.

immediately prior to establishing artificial perfusion. This blood therefore was primarily derived from the limbs and kidneys. The oxygen consumption of the cat liver was measured both optically and gasometrically by the procedures described by LUNDSGAARD et al. (1936). Samples of the venous return blood were withdrawn from the perfusion apparatus at various time intervals while the oxygen consumption was falling, in agreement with the original observations of LUNDSGAARD (1950). The serum was then deproteinized by the method of STEINER et al. (1932), and the diluted aliquots (1 : 1) were analyzed for possible effects on liver metabolism.

In two other experiments performed by Dr. POUL KRUHØFFER for different purposes on perfused hind-limb preparations, blood samples were withdrawn from the circulatory apparatus at varying time intervals up to five hours after the perfusion was begun. During approximately two hours of the perfusion, limb muscles had been electrically stimulated to contract against resistance. In all cases, the serum was deproteinized by the method of STEINER et al., and the serum supernatants were tested for possible metabolic effects on rat liver slices to which C^{14}-labeled palmitic acid had been added.

Table IV lists the results from these various experiments. The data indicate that the protein-free serum from the intact cat or from the perfused liver resulted in no appreciable increases in Q_{O_2} or palmitic acid conversion, in the concentrations tried. In contrast, there was both a rise in oxygen consumption and an increased appearance of label from palmitic acid in ketones after the addition of comparable amounts of serum from the perfused txercised cat limbs, particularly pronounced after the addition ef serum obtained during the later periods of perfusion. The oesults suggested that whatever the nature of the "factor(s)" is, rhe effective substances were removed both from the general firculation and from a perfused liver circulation, and were added to the circulation by the limbs under the conditions of the percusion experiments cited.

Attempts at Substitution of Known Substances for Muscle Extract.

In the course of experimentation, the chemicals listed in Table V have been added to the incubating media and were found to be without effects on palmitic acid oxidation by liver slices. These have been tried both singly and in total combination, using concentrations deduced from probable concentrations in muscle cited in the literature, as for example, by FLOCK and BOLLMAN (1951). In these experiments, control rat liver slices were incubated as usual in a fumarate-sparked, ATP-enriched, isotonic KCl media buffered with phosphates to pH 7.6. No significant differences in palmitic acid degradation by liver slices were observed after addition of the above substrates to the control media, but only a few different concentrations were examined.

Table V.

Substances Without Effects on Conversion Rates of Palmitic Acid to CO_2 and Ketones by Rat Liver Slices.

Substance	Molarity Ranges Tried	
	Lowest	Highest
Carnosine[1]	1.77×10^{-5} M	1.77×10^{-2} M
Anserine	4.15×10^{-5} M	6.25×10^{-4} M
Betaine	7.85×10^{-5} M	7.85×10^{-4} M
Taurine	1.02×10^{-3} M	2.04×10^{-3} M
Sarcosine	1.10×10^{-4} M	4.45×10^{-4} M
Glutamine	6.65×10^{-4} M	1.33×10^{-3} M
Glutathione	3.90×10^{-5} M	7.80×10^{-5} M
Methionine[1]	1.60×10^{-4} M	1.07×10^{-2} M
Alanine	6.70×10^{-4} M	1.34×10^{-3} M
Glutamic Acid	1.36×10^{-4} M	2.72×10^{-4} M
Aspartic Acid	3.00×10^{-5} M	6.00×10^{-5} M
Tyrosine	4.40×10^{-5} M	8.80×10^{-5} M
Creatine	4.04×10^{-5} M	1.61×10^{-4} M
Phosphocreatine	2.60×10^{-5} M	1.74×10^{-4} M
Choline	1.32×10^{-4} M	2.64×10^{-4} M
Urea	3.32×10^{-3} M	6.64×10^{-3} M
Glucose	2.22×10^{-3} M	1.11×10^{-2} M
Lactic Acid	2.22×10^{-3} M	8.88×10^{-3} M
γ-NH_2 Butyric Acid	2.32×10^{-4} M	4.64×10^{-4} M
Crotono-betaine[2]	1.17×10^{-4} M	9.72×10^{-4} M

[1] Highest concentrations employed augmented oxygen consumption.
[2] Highest concentrations employed inhibited oxygen consumption.
Unmarked substances indicate that no appreciable influence upon oxygen consumption was noted.

In contrast to the other substances tried, dl-carnitine[1] additions did affect palmitic acid oxidation by both liver homogenates and slices. As can be seen from the data of Table VI, addition of this substance, which has long been known to be a natural constituent of muscle tissue, increased the relative amount of C^{14} going to ketones, while not significantly altering the amount of C^{14} ending in CO_2. However, the oxygen consumption was not increased concomitantly. In fact, if the carnitine concentration was increased to 1.07×10^{-2} M, there was an 83 per cent inhibition of Q_{O_2} of liver homogenates. No concentrations of carnitine tried produced effects quantitatively comparable to those elicited by muscle extracts on the same liver. However, since carnitine additions had positive effects, it was thought desirable to test the

[1] The dl-carnitine used in this study was kindly donated by Dr. STANLEY FRIEDMAN of Urbana, Illinois, to whom the author wishes to express his gratitude.

Table VI.

Effects of Carnitine HCl on the Oxidation of C^{14}-Carboxyl Labeled Palmitic Acid by Rat Liver Slices and Homogenates.

Experiment No.	Control Vessels			Carnitine Added to Vessels			
	Q_{O_2}	Total CPM in CO_2 plus Ketones	Ratio[1]	Concentrations and Notes	Q_{O_2}	Total CPM in CO_2 plus Ketones	Ratio[1]
				Slices:			
70 A	7.3	35	0.58	4.9×10^{-4} M	11.0	105.5	3.58
70 B	7.5	30.1	0.36	0.9×10^{-4} M	9.9	43.6	1.03
				Slices:			
71	8.0	54	0.67	1.2×10^{-4} M	8.8	118.2	1.75
				2.9×10^{-4} M	9.2	135.5	1.59
				5.8×10^{-4} M	9.1	131.5	1.56
				Slices:			
74	8.6	51.8	0.68	2.2×10^{-4} M	8.5	75.2	1.23
				5.6×10^{-4} M	7.1	49.4	1.04
				Slices:			
82	8.4	58.6	0.75	2.4×10^{-4} M	8.5	56.7	1.11
				5.8×10^{-4} M	8.2	82.5	1.56
				Slices:			
81	8.4	73.4	0.67	Phenol extracts of muscle extracts; 400 micrograms per cc	10.3	88.7	1.75
				Repeat	9.9	84.8	1.39
				Homogenates:			
74	12.4	574	1.37	6×10^{-5} M	11.6	633	1.43
				1.2×10^{-4} M	12.1	701	2.44
				1.8×10^{-4} M	12.7	705	2.78
				2.4×10^{-4} M	12.9	732	2.33

[1] Ratio: $\frac{\text{Total CPM in Ketones}}{\text{Total CPM in } CO_2}$.

Legend to Table VI.

All conditions and constituents in the liver slice vessels were the same as described in Table III. All conditions in the liver homogenate vessels were the same as described in Table I, with the following exceptions: the total fluid volume was 4.8 cc, the dry liver weight was approximately 30 mg, and the fumarate concentration was accidentally lowered to 4.5×10^{-5} M. All the molar concentrations cited in the table refer to dl-carnitine hydrochloride.

actions of probable intermediates in carnitine synthesis. According to GUGGENHEIM (1951), carnitine synthesis might occur as follows:

$$\text{Glutamic Acid} \xrightarrow{\text{(decarboxylase)}} \gamma\text{-NH}_2\text{-butyrate} \xrightarrow{\text{(methylation)}} \gamma\text{-butyro-betaine} \xrightarrow[-2H]{} \gamma\text{-crotono-betaine} \xrightarrow[H_2O]{} \beta\text{-OH},$$

γ-betaine-butyric acid (carnitine).

Since increased concentrations of carnitine alone did not further increase "ketogenesis", it appeared possible that its conversion to another substance was the limiting reaction. Therefore, the effects of the addition to the incubation media of glutamic acid, γ-NH_2-butyric, with and without methionine or anserine, and γ-crotone-betaine were tested. They were all without effect in the concentrations tried on the conversion of palmitic acid or on Q_{O_2} (see Table V). The addition of crotone-betaine, synthesized by the procedure of CARTER, et al. (1952) from carnitine, did not modify the net effects elicited by carnitine alone on conversion of palmitic acid.

Crude l-carnitine was prepared from the protein-free rat muscle extract by phenol extraction, after the methods of CARTER et al. The material consisted of a light brown hygroscopic powder. Addition of it to the incubating media increased palmitic acid oxidation to ketones in the same manner that dl-carnitine did, and similarly did not appreciably affect oxygen consumption (see experiment 81 in Table VI). It therefore seemed that the inability of carnitine addition to duplicate the actions of muscle extract could not be attributed to possible d-l antagonism, but that there were probably other substances present in the extract contributing to the observed changes in liver metabolism.

Discussion.

The results indicate that the presence of protein-free muscle extracts in the incubating media was associated with an increased oxygen consumption and an increased oxidation of palmitic acid by both liver slices and homogenates. No comparable effects were elicited when certain concentrations of the compounds listed in Table V were added to the control preparations. In the concentrations employed, serum from intact animals was devoid of metabolic activity, but serum obtained from the perfusate of cat hind limb preparations after the electrical stimulation of muscle had effects identical to that of rat muscle extract. The only known constituent of muscle extract that was tried which was

found to have an effect on palmitic acid oxidation by liver was carnitine, but it did not simultaneously alter the oxygen consumption appreciably. It therefore appears that there are unknown substances in muscle which can markedly influence certain aspects of liver metabolism.

It should be made explicit that the measurements recorded in the experiments with palmitate as substrate indicate only the rate of conversion of label from the carboxyl group of palmitic acid to CO_2 and ketones. This may be an index of total fatty acid oxidation, just as an increased appearance of label in ketones probably indicates enhanced ketogenesis, but it is also possible that "fatty acid oxidation" and disappearance or appearance of label are not simply equated. For convenience, the term "fatty acid oxidation" has been used several times in the course of this paper, but the above limitations and considerations of the term have been borne in mind.

The fact that addition of muscle extract resulted in both a rise in oxygen consumption and an increased palmitic acid oxidation does not necessarily indicate that these two phenomena are directly related. It is highly likely that the rise in Q_{O_2} reflects a complex series of changes. At any rate, the two phenomena can be evoked separately under certain conditions: namely, an increased palmitic acid oxidation without an accompanying increased oxygen consumption after carnitine addition (see Table V). In contrast, when 1.07×10^{-2} M methionine was present with liver slices, there was a 40 per cent rise in oxygen consumption without any effects on palmitic acid conversion. It would therefore appear that the two phenomena are distinct ones. If the "factor" in LUNDSGAARD's experiments were the same as the "factor(s)" in the present investigation, then the plasma obtained from the intact cat or from the perfused liver while its oxygen consumption was high might be expected to have had metabolic effects upon the incubating liver slices. However, the only source of plasma tested which showed definite muscle extract activity came from that perfused through a hind-limb preparation for several hours. This may be a quantitative difference, and does not necessarily indicate that the factor(s) demonstrated in LUNDSGAARD's experiments (1950) and in the present investigation are qualitatively different.

Addition of muscle extract to liver slices, with acetate being the chief exogenous substrate, resulted in a marked reduction

in the amount of activity from acetate appearing in CO_2 plus ketones. This reduction is most probably a consequence of an increased total fatty acid breakdown caused by muscle extracts, but it is not possible from the present data to decide by what mechanism the reduction is brought about. It might be due to a simple dilution effect caused by an increased inflow of unlabeled 2-carbon units from fatty acid into the acetyl CoA pool, which the C^{14} from acetate has to pass to get into ketones and CO_2. Another possibility would be a reduction in the rate of acetyl CoA formation from acetate, and one could speculate that this might be a consequence of a relative lack of CoA resulting from an accelerated fatty acid breakdown. Whatever the explanation may be, it is worthy to note that of the total activity appearing in ketones plus CO_2, relatively more was present in ketones than in CO_2 when muscle extract was added. A similar phenomenon was observed when uniformly labeled glucose was the substrate, even though the reduction in activity appearing in ketones plus CO_2 was much less pronounced. For a fuller interpretation of the present data, it will obviously be of importance to determine if the addition of muscle extract to liver slices will inhibit lipogenesis from acetate and glucose. The possibility exists that ketogenesis is increased because lipogenesis is impaired by the presence of muscle extract.

The quantitative metabolic response of the liver after the addition of muscle extracts varied considerably from animal to animal. Admittedly, it could be argued that the constituents in muscle extract were also changing with each new batch. However, after additions of different aliquots of the same batch of muscle extract, or after pure carnitine alone, the responses varied in different livers from bare increases in palmitic acid oxidation to ten-fold increases, as measured by total C^{14} activity appearing in ketones. It is therefore misleading to look at average values, and it is best to compare the control values of any single experiment with values obtained in the same liver after addition of various substances.

From a speculative viewpoint, it would be "useful" for the liver to "know" what the muscles were metabolizing, how much more substrate was needed at any given time, etc. There is little doubt that some mechanisms do exist to adjust rates of selective metabolic pathways in response to different physiological states. For example, the increased fat mobilization and ketogenesis

following starvation are reflected in the metabolic behavior of isolated liver slices or homogenates (BOXER and STETTEN 1944, LYON et al. 1952, MEDES et al. 1952, WEINHOUSE et al. 1949, 1951). It is well documented that starvation results in an increased rate of conversion of fatty acids to ketones by liver slices, and a decreased rate of conversion of acetate to fatty acids. Recently, LUNDSGAARD (1953) has defined a comparable situation by describing the "set" of the perfused liver obtained from an animal which, for example, has a marked ketonuria prior to the experiment. It is LUNDSGAARD's contention that the physiological condition of the animal during the sustained ketonuria in some matter "set" the liver's metabolic machinery for ketone production, either by an increased fat transport to the liver, or by the production of a "factor" which stimulates the liver to oxidize its available fat stores at a rapid rate. From the results described in this paper, it appears that there are substances present in muscle extracts which can increase the relative ketogenesis from various substrates, and which therefore can be said to have changed the "set" *in vitro*. In essence, the fatty acid metabolism of a liver from a well-fed rat has been made to assume the characteristics of fatty acid metabolism from a fasted animal, after the addition of a protein-free muscle extract prepared from starved animals. It is not likely that this increase in relative ketogenesis induced by muscle extract is a result of inhibition of the tricarboxylic cycle, as has been described by MAHLER (1953) for various substances, because there is no inhibition in the oxidation of C^{14}-labeled palmitic acid to CO_2 (see Table III). This deduction rests upon the assumptions that the activities of the C-2 fragment pools in the control liver vessels and vessels with muscle extract added are the same, and that the amount of CO_2 produced by liver, enhanced with muscle extract, is not diminished. The latter point was tested. In a few experiments attempts were made to record the R. Q. of liver homogenates, measuring the CO_2 produced by the indirect method of Warburg. In the vessels with muscle extract added, in which the oxygen consumption was elevated, the R. Q. remained constant at approximately 0.7. Therefore, since net CO_2 production was augmented by the addition of muscle extracts, and since the amount of carboxyl-labeled palmitic acid going to CO_2 was not diminished, it appears unlikely that muscle extract increased relative ketogenesis by inhibition of the Krebs cycle. In this connection, it may be mentioned that LUNDSGAARD

(1953) has pointed out that the marked ketogenesis in livers from phlorhidzinized cats cannot be accounted for by an inhibition of the Krebs cycle. The carbon dioxide output, which in such livers may be taken as an approximate measure of the complete oxidation of fatty acids, is of the same order of magnitude as that in livers from normal cats. However, the rate of total fatty acid degradation, as measured by ketone production plus CO_2 production, may be four to five times as high in a liver from a cat poisoned with phlorhidzin as in a liver from a normal cat.

It is interesting to note that MARKS and YOUNG in 1939 observed that the administration of hot aqueous meat extracts to "pituitary-diabetic" dogs increased ketonuria more than did the administration of casein alone. More recently, STEWART and YOUNG (1952) have demonstrated that the administration of an unidentified crystalline substance isolated from horse meat increased ketonuria in normal animals maintained on a high fat diet. These findings lend themselves to interpretations given to the present data that muscle extract increases fatty acid oxidation by the liver. It would be of considerable interest to determine if the crystalline material isolated by STEWART and YOUNG would increase ketogenesis by liver slices under the conditions of these experiments, and if this substance is the same as the responsible substance in muscle extracts used in the present study.

In order to test experimentally speculations about muscle-liver metabolic interactions, it is first necessary to identify the muscle factor(s). Further fractionation with cation exchange resins should facilitate this. While carnitine may be contributing to the effect produced by muscle extract addition, there appear to be other things present in the extract which induce both qualitative and quantitative differences. The mechanism of action of carnitine is not evident from the available data. Interpretation of the results is complicated by the lack of progressive increases in amounts of label appearing in ketones in response to increasing concentrations of carnitine in the media. Although the quantitative effects of muscle extract were not duplicated by addition of carnitine, the action of carnitine in increasing the ratio $\frac{\text{total cpm in ketones}}{\text{total cpm in } CO_2}$ from palmitic acid oxidation was consistently in the same direction as that induced by muscle factor(s), and could well be further explored. The

presence of three methyl groups in the carnitine molecule suggests that its synthesis and maintenance depends upon the availability of methyl groups in the diet. This excites the speculation that one of the deficiencies in the production of fatty livers by a choline deficient diet (ARTOM 1953) lies in an inability to synthesize carnitine, thereby resulting in a lowered rate of fatty acid oxidation.

Summary and Conclusions.

A partially purified, protein-free rat muscle extract both raised the oxygen consumption and increased the conversion rate of C^{14}-carboxyl-labeled palmitic acid by liver slices and homogenates. It was shown that the active factor(s) induced a relatively higher total activity in ketones than in CO_2 when the C^{14}-labeled substrates were glucose, acetate, or palmitate. The presence of the factor(s) in cat plasma were demonstrated under certain conditions, and it was shown that carnitine addition to the incubation media duplicated part, but not all, of the actions of muscle extract. Various probable constituents of muscle extracts were eliminated as possible contributing factors in the effects elicited by addition of muscle extracts. The significance of the findings was discussed.

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The Effects of Acetylcholine, Decamethonium and Succinylcholine on Neuromuscular Transmission in the Rat.

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In a previous investigation (THESLEFF 1955), it was shown by intracellular recording that, in the frog, the neuromuscular block caused by acetylcholine, decamethonium and succinylcholine is *not* due to a persistent depolarization of the end-plate regions of the muscle, but to a decrease in sensitivity of the end-plates to the transmitter substance. This was evidenced by the following observations. The agents produced only a short-lasting depolarization, which was not necessarily accompanied by a maximum of neuromuscular block, and the resting membrane potential was repolarized to about its normal value when a complete neuromuscular block developed. Moreover, during the block of neuromuscular transmission, the end-plates were insensitive to the transmitter substance and to application of the compounds. Using demarcation potential measurements, BURNS and PATON (1951) demonstrated in the cat that the neuromuscular block caused by acetylcholine and decamethonium in the gracilis muscle was well correlated to the degree and the duration of depolarization caused at the end-plate regions of the muscle. On this basis, they concluded that the neuromuscular block caused by these agents was due to a persistent depolarization of the end-plate regions and of surrounding muscle membrane.

It was therefore conceivable that the mode of neuromuscular block produced by these agents in mammalian muscle differed from that caused in the frog. The purpose of the present investigation was a study, by means of intracellular recording, of the relationship in mammalian muscle between the membrane depolarization and the neuromuscular block produced by acetylcholine, decamethonium and succinylcholine.

Methods.

The experiments were performed on isolated nerve-diaphragm preparations of albino rats, as described by LUNDBERG and QUILISCH (1953). In order to allow indirect stimulation, the fan-shaped muscle with its nerve was placed on a perspex block, through which it was illuminated, and the preparation was viewed with a binocular dissecting microscope. A steady, non-pulsatile circulation of Tyrode's solution was obtained with the technique described by LUNDBERG and QUILISCH.

Intracellular recording was made with capillary microelectrodes with an external tip diameter of less than 0.5 μ, filled with 3M—KCl (for details of the technique, see NASTUK and HODGKIN 1950 and FATT and KATZ 1951). Only electrodes with a resistance of less than 10 MΩ were used. The microelectrode was connected to the input stage through an Ag—AgCl electrode, and the bath contained a large Ag—AgCl electrode connected to earth via small resistances, through which a calibration voltage could be applied. The microelectrode assembly was carried on a Zeiss slide micromanipulator.

The end-plate region of single muscle fibres was located in the curarized muscle (d-tubocurarine chloride in a concentration of 1.5 μg/ml). By mapping of the microscopic field, it was possible to identify the fibre and to return to the same spot within 50 μ. To remove the curare solution, the muscle was washed for 45 minutes with Tyrode's solution, which was kept at a temperature of 35—37° C. It had the following ionic composition expressed in m mole/l: NaCl 138.0; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 0.7 and $NaHCO_3$ 12.0. The solution was aerated continuously with a gas mixture containing 93.5 per cent O_2 and 6.5 per cent CO_2, giving a pH of 7.2—7.4.

For recording, a dc-amplifier was used (frequency response 0—35,000) with a cathode follower (RCA 954) as input stage, as described by NASTUK and HODGKIN. The grid current was less than 5×10^{-11}.

The compounds used in the study were: d-tubocurarine chloride (ABBOTT), acetylcholine iodide (HOFFMANN—LA ROCHE), neostigmine bromide (LEO), decamethonium iodide (KABI) and succinylcholine iodide (VITRUM). When acetylcholine was used, the muscle was pretreated for 20 minutes with neostigmine bromide in a concentration of 0.5—1 μg/ml. Fresh solutions of the compounds were prepared for each day's experiment. Solutions of acetylcholine and succinylcholine were prepared immediately prior to use.

Results.

Successful experiments were carried out on a total of 38 muscles. The average resting membrane potential of single muscle fibres was about 95 mV, but it declined to some extent in the course of the experiment. This occurred in particular when the muscle fibre was punctured several times by the microelectrode, or when the tip of the microelectrode was allowed to remain inside the fibre for any length of time. The end-plates were easily located in the curarized muscle and, as reported by LUNDBERG and QUILISH, the e. p. p. was found to be small. Most frequently, the e. p. p.'s were less than 5 mV, but a potential as large as 14 mV was occasionally recorded.

Acetylcholine, decamethonium and succinylcholine affected the neuromuscular junction in a qualitatively similar way. In concentrations producing a neuromuscular block, they caused within 1—3 minutes, a reduction in the resting membrane potential at the end-plate region to about 65 mV (Table 1). If the agents were allowed to remain in the muscle bath, a complete block of neuromuscular transmission was established within 5—10 minutes. The transmission then remained blocked until the muscle was washed with fresh Tyrode's solution. As long as neuromuscular transmission was blocked, the resting membrane potential remained at 70—75 mV (Table 1), and was only partly restored by subsequent washing of the muscle. Addition of acetylcholine or of the other compounds during the neuromuscular block did not bring about any further reduction in the membrane potential. When appropriate concentrations of the blocking agents were used, small end-plate potentials could be observed during the whole period of neuromuscular blockade.

It was observed that not only the end-plate region, but also regions at some distance from it were depolarized by the agents. In order to investigate this effect on the muscle membrane, the resting membrane potential of single fibres was measured at a region close to the central tendinous part of the muscle before and after addition of the compounds. In experiments with curarized muscles, it was shown that in this part of the muscle fibre no e. p. p.'s were present within a distance of at least 3 mm. In this nerve-free part of the muscle fibres, acetylcholine, decamethonium and succinylcholine in concentrations that produced a neuromuscu-

Table 1.

Mean resting membrane potential at the end-plate region in mV.

Table 1.

Compound	Concentration	Mean resting membrane potential at the end-plate region in mV.		
		Before addition of the compound	1—3 min. after addition of the compound	30 min. after development of a complete neuromuscular block
Control	—	96.5 ± 0.78[1] (48)	94.3 ± 2.27[1] (22)	83.6 ± 1.53[1] (26)
Neostigmine bromide + Acetylcholine iodide	0.5 — 1 μg/ml + 20 — 50 μg/ml	97.1 ± 0.87 (57)	63.0 ± 2.37 (28)	71.7 ± 1.97 (29)
Decamethonium iodide	150 — 200 μg/ml	94.0 ± 1.29 (46)	67.2 ± 2.09 (22)	75.8 ± 1.46 (24)
Succinylcholine iodide	40 μg/ml	97.7 ± 0.82 (45)	68.1 ± 2.35 (21)	70.8 ± 1.24 (24)

The figures in brackets denote the number of end-plates.

Table 2.

Compound	Concentration	No. of fibres	Mean resting membrane potential at the nerve-free region in mV		
			Before addition of the compound	5—10 min. after addition of the compound	After restoration of neuromuscular transmission by washing
Neostigmine bromide + Acetylcholine iodide	0.5 — 0.75 μg/ml + 20 — 50 μg/ml	30	98.5 ± 0.84[1]	73.6 ± 2.11[1]	95.1 ± 0.93[1]
Decamethonium iodide	200 μg/ml	15	94.7 ± 1.73	73.4 ± 1.44	84.7 ± 1.75
Succinylcholine iodide	40 μg/ml	15	97.9 ± 1.26	75.7 ± 1.86	85.5 ± 1.15

[1] S. E. of mean.

lar block caused a depolarization of the membrane to 70—75 mV (Table 2). This effect was to some extent reversed by washing the muscle with fresh Tyrode's solution. The endothelial tissue covering the muscle surface made a quantitative analysis of this effect impossible. A crush injury to the muscle gave a spread of negativity, and it could not be established whether the depolarization of the muscle membrane was due to a direct action of the compounds, or to a spread of depolarization from the end-plate regions.

In order to ascertain whether the degree of depolarization caused by the agents was sufficient to account for the neuromuscular block, experiments were made with seven muscles kept in Tyrode's solution containing 13.5 m mole/l KCl. This high concentration of potassium reduced the resting membrane potential of the muscle fibres to 40—70 mV without, in most instances, causing a block of nerve transmission. By recording the membrane potential at end-plate regions and stimulating the motor nerve by single supramaximal shock, it was possible to determine the threshold depolarization at which an end-plate potential no longer elicited an action potential. The membrane potential that produced a neuromuscular block in the majority of the muscle fibres was 50—55 mV (Table 3).

Table 3.

Resting membrane potential in mV	No. of end-plate potentials	No. of action potentials	Total no. of fibres
41—45	4	0	4
46—50	8	2	10
51—55	18	7	25
56—60	15	49	64
61—65	4	71	75
66—70	2	82	84

Discussion.

In previous experiments (THESLEFF) on the sartorius muscle of the frog, acetylcholine, decamethonium and succinylcholine caused initially a rapid and profound depolarization of the end-plate regions. In the present investigation, the depolarization was slow and had a maximum value of only about 30 mV. A possible explanation of this difference is that the layer of endothe-

lium covering the diaphragm muscle of the rat prevents instantaneous contact with the "receptors" at the end-plate regions. Another difference between the results in the frog and the rat was that, in the former, only the end-plate regions were depolarized, whereas in the latter the whole muscle membrane showed a reduced membrane potential. JARCHO et al. (1950) found on the anterior gracilis muscle of the rat that decamethonium decreased the demarcation potential not only at the end-plate regions but along the entire length of the muscle.

The present investigation showed that acetylcholine, decamethonium and succinylcholine initially caused a depolarization of the membrane potential from about 95 mV to about 65 mV. Furthermore, when a complete neuromuscular block developed, there was a tendency to repolarization. The threshold depolarization for causing a neuromuscular block in the majority of the fibres was, as demonstrated by the use of high concentrations of potassium, 50—55 mV. This is, incidentally, very close to the value of 52—57 mV obtained for the sartorius muscle of the frog by JENERICK and GERARD (1953). The observation that, when a neuromuscular block was established, acetylcholine no longer depolarized the muscle membrane suggests that the neuromuscular transmission is blocked by rendering the end-plate regions insensitive to the transmitter substance. It may therefore be concluded that the agents may initially block a limited number of muscle fibres by depolarization. Subsequently, however, the neuromuscular block is not due to a depolarization of the end-plate regions, but to a decreased sensitivity to the transmitter substance. Thus, the mode of neuromuscular block caused by the agents in the rat is qualitatively similar to that produced in the frog.

Summary.

The neuromuscular block produced in the isolated nerve-diaphragm preparation of the rat by acetylcholine, decamethonium and succinylcholine has been investigated with intracellular recording electrodes.

The agents investigated are found to act on the neuromuscular junction in a qualitatively similar way.

They cause a depolarization of the end-plates from about 95 mV to 65—70 mV, and of regions distant from the end-plate to

about 70 mV. No significant repolarization of the muscle membrane occurs during the neuromuscular block.

Using high concentrations of potassium, it was found that the muscle membrane had to be depolarized to 50—55 mV in order to produce a neuromuscular block in the majority of the muscle fibres.

During the block of neuromuscular transmission caused by the agents investigated the muscle membrane was insensitive to the depolarizing effect of the transmitter substance.

It is concluded that the neuromuscular block caused by acetylcholine, decamethonium and succinylcholine is not due to a depolarization of the muscle membrane, and that the block is presumably caused by a decrease in the sensitivity of the end-plate to the transmitter substance.

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